The Role of Telomeric DNA Damage Response RNAs in Alternative Lengthening of Telomeres

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The Role of Telomeric DNA Damage Response RNAs in Alternative Lengthening of Telomeres

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The Alternative Lengthening of Telomeres (ALT) pathway, active in 10-15% of cancers, is characterized by homologous recombination (HR)-dependent telomere lengthening. Telomeres consist of hexameric TTAGGG repeats, which makes them difficult to replicate, and are prone to DNA replication stress. ALT cells display elevated basal levels of replication stress at their telomeres, and this damage has been intimately linked to the ALT phenotype. We recently discovered that non-coding RNAs transcribed de novo at sites of damage, including at telomeres, support local DNA damage signaling and repair. Inhibition of telomeric non-coding RNAs (tncRNAs) with antisense oligonucleotides (ASOs) leads to a reduction in DNA damage responses at uncapped telomeres in cultured cells, as well as in vivo. To further investigate the role of tncRNAs, we quantify their levels in ALT cells and find them to be more highly expressed than in non-ALT cells. We then inhibit tncRNA function with ASOs to further probe their role in ALT cells. Interestingly, inhibition of C-rich tncRNAs, transcribed from the TTAGGG repeats, with antiteloC ASOs induces apoptosis and reduces cell number in ALT-positive cells, but does not affect telomerase-positive or mortal cells. AntiteloC ASOs also induce an S-phase accumulation and inhibition of DNA replication in ALT cells, and non-cycling ALT-positive cells display no sensitivity to antiteloC ASOs, suggesting a prominent role for tncRNAs in overcoming endogenous ALT replication stress. Hydroxyurea treatment, which induces replication stress at common fragile sites such as telomeres, induces tncRNA transcription as well as enhanced sensitivity to antiteloC ASOs both in ALT-positive and ALT-negative cells.

Taken together, these results suggest that tncRNA transcription is induced upon telomeric replication stress, that teloC species are important in the replication stress response, and that ALT cells are sensitive to antiteloC ASOs due to their high levels of endogenous replication stress.
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Published manuscripts

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<th>Description</th>
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<tbody>
<tr>
<td>2'-O-Me</td>
<td>2-O-Methyl</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53 binding protein 1</td>
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<tr>
<td>9-1-1</td>
<td>RAD9-RAD1-HUS1</td>
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<tr>
<td>a-NHEJ</td>
<td>Alternative non-homologous end joining</td>
</tr>
<tr>
<td>AA</td>
<td>Anachardic acid</td>
</tr>
<tr>
<td>ALT</td>
<td>Alternative Lengthening of Telomeres</td>
</tr>
<tr>
<td>APB</td>
<td>ALT-associated PML body</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
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<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
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<tr>
<td>ATR</td>
<td>ataxia telangiectasia and Rad3-related</td>
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<tr>
<td>ATRi</td>
<td>ATR inhibition</td>
</tr>
<tr>
<td>ATRIP</td>
<td>ATR-interacting protein</td>
</tr>
<tr>
<td>ATRX</td>
<td>A-thalassemia/mental retardation syndrome X-linked</td>
</tr>
<tr>
<td>BIR</td>
<td>Break-induced replication</td>
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<tr>
<td>BITS</td>
<td>Break-induced telomere synthesis</td>
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<tr>
<td>BLM</td>
<td>Bloom syndrome protein</td>
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<td>Breast cancer 1</td>
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<td>Breast cancer 2</td>
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<td>BTR</td>
<td>BLM, Topoisomerase IIIa, and RMII/2 complex</td>
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<td>CtBP interacting protein</td>
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<td>DAXX</td>
<td>Death-domain-associated protein</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<td>DNA damage response RNA</td>
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<td>dilncRNA</td>
<td>DNA damage-induced long non-coding RNA</td>
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<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
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<td>DSB</td>
<td>Double-strand break</td>
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<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
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<td>Histone 3 lysine 9 trimethylation</td>
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<td>Histone acetyltransferase</td>
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<td>HGPS</td>
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</tr>
<tr>
<td>hnRNPA1</td>
<td>Heterogeneous nuclear ribonucleoprotein A1</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>hTERC</td>
<td>Human Telomerase RNA component</td>
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hTERT  Human telomerase reverse transcriptase
IC50  Inhibitory concentration at which 50% of maximum effect is achieved
iDDR  Inhibitor of DDR domain in TRF2
IR  Ionizing radiation
KAT5  Lysine acetyl transferase 5
LMNA  Lamin A and C gene
LNA  Locked nucleic acid
m7G  7-methylguanosine
MALAT1  Metastasis-associated lung adenocarcinoma transcript 1
MDC1  Mediator of DNA damage checkpoint 1
MiDAS  Mitotic DNA synthesis
MRN  Mre11, RAD50, NBS1 complex
mRNA  Messenger RNA
ncRNA  non-coding RNA
NHEJ  Non-homologous end joining
PAGE  polyacrylamide gel electrophoresis
PALB2  Partner and localizer of BRCA2
PARP1  poly-ADP-ribose polymerase 1
PCAF  P300/CBP-associated factor
PCNA  Proliferating cell nuclear antigen
PML  Promyelocytic leukaemia bodies
PNA  Peptide Nucleic Acid
Pol λ  DNA polymerases λ
Pol μ  DNA polymerase μ
POT1  Protection of telomeres 1
progerin  Truncated form of Lamin A that results in HGPS
PS  Phosphorothioate
RFC  Replication factor C
RISC  RNA-induced silencing complex
RM1/2  RecQ-mediated genome instability protein 1/2
RNA pol II  RNA polymerase II
RNAi  RNA interference
ROS  Reactive oxygen species
RPA  Replication protein A
RPLPO  Ribosomal protein lateral stalk subunit P
RT  Room temperature
RT-qPCR  Reverse transcription quantitative PCR
SA-β-gal  senescence-associated-β-galactosidase
SLX1-4  Structure-specific endonuclease subunits 1-4
SMARCAL1  SWI/ SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1
smFISH  Single molecule FISH
SSB  Single-strand break
ssDNA  single-stranded DNA
t-circle  telomeric circle
t-loop  Telomeric loop
<table>
<thead>
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<th>Acronym</th>
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<tr>
<td>T-SCE</td>
<td>Telomeric sister chromatid exchange</td>
</tr>
<tr>
<td>tDDRNAs</td>
<td>Telomeric DNA damage response RNAs</td>
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<td>tdlncRNAs</td>
<td>Telomeric damage-induce long non-coding RNAs</td>
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<td>teloC</td>
<td>C-rich telomeric RNAs</td>
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<tr>
<td>teloG</td>
<td>G-rich telomeric RNAs</td>
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<tr>
<td>TERRA</td>
<td>Telomeric repeat-containing RNA</td>
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<tr>
<td>TEsR</td>
<td>Targeted enrichment of small RNAs</td>
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<td>TIF</td>
<td>Telomere dysfunction-induced foci</td>
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<td>TIN2</td>
<td>TRF1-interacting nuclear protein 2</td>
</tr>
<tr>
<td>TMM</td>
<td>Telomere maintenance mechanism</td>
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<tr>
<td>tncRNAs</td>
<td>Telomeric non-coding RNAs</td>
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1 Introduction
1.1 The DNA damage response

DNA damage can lead to genetic mutations, a cause of cellular dysfunction and cancer. Despite these severe consequences of DNA damage, every day the genome of each of our cells is assaulted by tens of thousands of lesions (Lindahl et al. 2000). Reactive oxygen species (ROS), which are produced during oxidative respiration and cause DNA oxidation (Valko et al. 2006), and ultraviolet (UV) light, which is produced by the sun and causes cross linking of DNA (Rastogi et al. 2010), are the most common endogenous and exogenous sources of DNA damage, respectively. To counteract this overwhelming amount of damage our cells have evolved a coordinated series of responses, known as the DNA damage response (DDR) (Harrison et al. 2006, Harper et al. 2007, Polo et al. 2011).

1.1.1 The DNA damage response pathway

Damaged DNA bases can be directly excised, thereby generating single-stranded breaks (SSBs) in the DNA duplex. If two SSBs occur in close proximity on complementary strands, a DNA double-strand break (DSB) can be generated, which is the most challenging for cells to repair (Khanna et al. 2001). Ionizing radiation (IR) can also directly cause DSBs. DSBs, marked by free DNA duplex ends, and SSBs induce activation of the DDR. The first step in the DDR is DNA damage sensing, followed by activation of the apical kinases ataxia telangiectasia and Rad3-related (ATR), ataxia telangiectasia mutated (ATM), or DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which then phosphorylate other DDR proteins, spreading the signal throughout the cell and recruiting various factors to the site of damage, and possibly triggering a cell cycle check point. The DDR concludes, when possible, with repair and eventual proliferation, and if repair is not possible with cellular senescence or apoptosis.

The three major DDR kinases ATM, ATR, and DNA-PK are members of the family of phosphatidylinositol 3-kinase-like kinases which phosphorylate targets on serine or threonine moieties, when followed by glutamine, also called S/TQ motifs (Bensimon et al. 2010). These three kinases define the DDR pathway trifurcation (Figure 1), and which kinase is activated is based on what type of damage is present, and what proteins sense the damage. A DSB can be sensed either by the Mre11, RAD50, NBS1 (MRN) complex, or by the KU70/80 heterodimer complex. KU70/80 heterodimer-sensing of DSBs recruits DNA-PKcs. Upon DSB localization, DNA-PKcs under goes autophosphorylation and thus activation. When DSBs are sensed by MRN, it recruits a monomer ATM, which allows for the acetylation of ATM by lysine acetyl transferase 5 (KAT5) (Sun et al. 2005). KAT5-mediated acetylation of ATM, thought to promote homodimerization from ATM monomers,
Adapted from (Blackford et al. 2017)

Figure 1. The DNA damage response mediated by DNA-PKcs, ATM, and ATR.

DSBs can be sensed by: KU70/80, activating DNA-PKcs; by MRE11-RAD50-NBS1, activating ATM; or RPA binding to ssDNA, activating ATR. All DDR pathways can lead to repair, cell cycle modulation, and eventually if the damage is not repaired senescence or apoptosis.

allows for autophosphorylation. Once homodimerized and autophosphorylated ATM is fully activated, then it phosphorylates other DDR factors, including a downstream protein kinase CHK2, triggering a signalling cascade leading to the focal recruitment of many ATM-dependent DDR factors such as breast cancer 1 (BRCA1), mediator of DNA damage checkpoint 1 (MDC1), and p53 binding protein 1 (53BP1) (Bekker-Jensen et al. 2005, Stucki et al. 2005). SSBs, instead, are sensed by replication protein A (RPA) coating of ssDNA, which recruits ATR-interacting protein (ATRIP) and thus ATR (Zou et al. 2003, Ball et al. 2005). Topoisomerase II binding protein 1 (TopBP1) and RAD9-RAD1-HUS1 (9-1-1) clamp complex are also recruited to SSBs, and allow for activation and spread of the ATR-mediated DDR signal (Cimprich et al. 2008). ATR phosphorylates the downstream protein kinase checkpoint kinase 1 (CHK1), and similar to ATM, signalling culminates in the focal recruitment of many ATR-dependent DDR factors.

The ATM and ATR signalling cascades share some common features, and there is also evidence of cross talk. For example, both ATM and ATR induce phosphorylation of the histone variant H2AX (phosphorylated form called γH2AX), which leads to a positive feedback loop propagating γH2AX along chromatin (Iacovoni et al. 2010), and also serves
as a nucleation point for the focal recruitment of DDR proteins (Martin et al. 2009). ATM and ATR also seem to recruit one another, as MRN together with CtBP interacting protein (CtIP) execute 3’ to 3’ resection thereby revealing single-stranded DNA (ssDNA) (Jazayeri et al. 2006), to which RPA binds and signals for ATR, while ATR-mediated phosphorylation of H2AX upon replication stress may recruit ATM (Ward et al. 2001). Thus, these two pathways overlap to a certain extent.

Repair of DSBs can proceed through two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) (Figure 2) (Chapman et al. 2012, Chang et al. 2017). While NHEJ is active throughout the cell cycle, it is the only repair

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**Figure 2. DSB repair by homologous recombination and non-homologous end joining**

HR: following MRN-mediated DSB sensing and minimal 3’ to 3’ resection, CtIP executes further resection, ATM and ATR signalling are activated, and RPA is loaded onto ssDNA; BRCA1 and BRCA2 induce a swap of RAD51 for RPA on ssDNA; RAD51-mediated strand invasion of a homologous sequence precedes DNA synthesis; HR concludes with ligation and Holliday Junction processing. NHEJ: following KU70/80-mediated DSB sensing, DNA-PKcs signalling is activated; Artemis minimally processes DNA ends to prepare for ligation; NHEJ concludes with XRCC3- and DNA Ligase IV-mediated ligation of DNA ends.
pathway active during G0, G1, and early S-phases of the cell cycle (Lieber 2010). HR, instead, is restricted to DSBs introduced during late-S or G2 (Karpenshif et al. 2012). This cell cycle distribution of repair pathways reflects the absence (G1) or presence (late-S/G2) of sister chromatids, and thus a homologous sequence with which to pair during HR. NHEJ repair is divided into classical NHEJ (c-NHEJ), and alternative NHEJ (a-NHEJ), also known as micro homology-mediated end joining. c-NHEJ, the most commonly used repair pathway, proceeds following KU70/80 recognition of a DSB, whereby DNA-PKcs is recruited in a complex with the endonuclease Artemis that performs minimal resection or ‘cleaning’ of the DNA end (Goodarzi et al. 2006, Gu et al. 2010). If DNA ends have a ssDNA overhang, DNA polymerases μ (Pol μ) and Pol λ (Moon et al. 2014) promote strand fill-in, followed by DNA ligase IV-mediated joining of the ends (Chang et al. 2017). On the other hand, the less-characterized a-NHEJ seems to require a minimal amount of homology between the DNA ends that are to be ligated, between 2 bp and 20 bp, and may be dependent on MRN-induced resection rather than KU70/80 sensing of DNA ends, but seems to only become relevant when NHEJ is compromised (Chang et al. 2017). If MRN-initiated resection of DSBs leads to RPA coating of the 3′-end of ssDNA, however, the DSB will be repaired by HR. RPA is replaced on ssDNA by RAD51 through the actions of HR mediators such as breast cancer 2 (BRCA2) and RAD52 (Zelensky et al. 2014). RAD51-coated ssDNA then invades and complementarily binds to a homologous DNA, generating a displacement loop (D-loop) that pairs with the other exposed 3′end of ssDNA, and thereby exchanging strands. Following D-loop formation and strand exchange, the 3′-ends are extended by DNA polymerases dependent on the activity of the ATP-dependent DNA translocase RAD54 (Li et al. 2009). Finally, the newly synthesized products of HR are ligated to close the DSB.

D-loop formation and strand exchange intertwine the DNA both at the site of 3′-end invasion, as well as the ligation of the newly-extended 3′-end back to the 3′-end of the DSB, thereby forming a double Holliday Junction. These junctions are most commonly dissolved by the Bloom syndrome protein (BLM) helicase together with topoisomerase IIIa, and RecQ-mediated genome instability protein 1 and 2 (RMI1/2), collectively known as the BTR complex, in which no crossover occurs, but can also be resolved either by Structure-Specific Endonuclease Subunits 1-4 (SLX1-4) together with the Crossover Junction Endonucleases MUS81-EME, or by the Flap Endonuclease Gen1, in which crossover and no crossover can occur (Figure 3) (Wyatt et al. 2014).
Dissolution and resolution of double Holliday Junctions

Dissolution of double Holliday Junctions is mediated by the BLM-Topo IIIa-RMI1/2 (BTR) complex leading to no crossover events. Resolution of double Holliday Junctions is mediated by either SLX4-MUS81-EME1-SLX1 or GEN1, and can result in either cross over or no cross over events.

1.1.2 Response to replication stress and break-induced replication

Replication stress is the general term for when a replication fork slows or pauses, and can be caused by a variety of potential replication barriers that include, but are not limited to: unrepaired DNA damage, secondary DNA structures such as G-quadruplexes, repetitive sequences, misincorporation of ribonucleotides, limiting nucleotide pools, and RNA-DNA hybrids (Zeman et al. 2014). Common fragile sites (CFS) are genomic loci inherently sensitive to replication stress, and thus commonly appear broken on metaphase spreads and rearranged in cancers (Hickson et al. 2018). Replication stress is characterized by the uncoupling of replicative helicases from the stalled polymerase, or fork slowing and pausing, which both result in the exposure of ssDNA (Pacek et al. 2004) which is recognized by RPA (Byun et al. 2005) and thus activates local ATR signalling (Figure 4a).
Adapted from (Zeman et al. 2014)

Figure 4. The replication stress response

(a) In response to ssDNA associated with replication stress RPA activates ATR, which in turn repair, and restart. (b) Stalled forks can be rescued by an incoming fork, or firing of a nearby dormant origin (left) or by fork reversal, and eventual rescue (right). (c) If not rescued stalled forks can collapse, caused by DSBs or replisome dissociation.

The main functions of ATR signalling at stalled forks seem to be activating a checkpoint and suppressing late origin firing by activating CHK1 in order to provide the cell ample time to repair the cause of fork blockage. Importantly, the signalling and repair processes that occur at sites of replication stress are similar to those at SSBs or DSBs, however given the unique architecture of a stalled fork there are distinct differences. One way that to restart replication at stalled forks starts with fork reversal, in which nascently replicated DNA anneals to each other, known as a ‘chicken foot,’ forming a Holliday junction (Sogo et al. 2002), which can be stabilized by ATR, and eventually restarted (Couch et al. 2013). A stalled replication fork can also be bypassed through the firing of a nearby dormant origin (Ge et al. 2007) or by a converging fork which then replicates through the stalled fork. A ‘collapsed fork’ occurs if the replisome machinery is lost, or a stalled fork is not promptly repaired and is processed into a DSB (Zeman et al. 2014). A collapsed fork with a DSB can potentially be rescued through break-induced replication (BIR), which is the repair pathways cells activate in response to single-ended DSBs.

BIR involves the invasion of a 3′-end of ssDNA into a homologous duplex, forming a holliday junction, and extension to the end of the chromosome (Kramara et al. 2018). BIR has mainly been characterized in prokaryotes, where there is a single origin of replication and smaller chromosomes thereby allowing the possibility that a single BIR event could complete an entire round of replication, whereas in eukaryotic cells it is thought that the presence of multiple replication origins allows for a converging fork to stop BIR prior to
replication termination at chromosome termini (Mayle et al. 2015). However, evidence points towards BIR as a source of genome duplication and rearrangement in mammals (Costantino et al. 2014), suggesting that BIR-initiated replication forks can reach the end of the chromosome. BIR-mediated replication is conservative (Saini et al. 2013), meaning that the newly-synthesized 3′-ended strand is then used as a template to fill in the complementary strand. Interestingly, BIR in mammals seems to be dependent on RAD52 rather than RAD51 (Bhowmick et al. 2016, Sotiriou et al. 2016). Mounting evidence points towards BIR as a central part of the Alternative Lengthening of Telomeres (ALT) pathway (Dilley et al. 2016), by which some cancer cells elongate their telomeres. The link between BIR and ALT will be explored in section 1.3.

1.1.3 A role for non-coding RNAs at DSBs

A conserved role for non-coding RNAs (ncRNA) in the DDR has become apparent in recent years (d'Adda di Fagagna 2014). Our group has recently characterized ncRNAs induced at DSBs, and uncovered their importance to both DDR signalling and repair of the DSB (Figure 5). Specifically, we discovered that upon sensing and localization to a DSB, MRN recruits RNA polymerase II (RNA pol II), which then transcribes both from and towards the DSB, thereby generating DNA-damage induced long non-coding RNAs (dilncRNAs) with the sequence of the damage locus (Michelini et al. 2017) up to 2kb in length (unpublished observations). Complementary dilncRNAs can then bind together, and are processed by the RNA interference (RNAi) machinery DROSHA and DICER into double-stranded DNA damage response RNAs (DDRNAs), 20-35 nt in length (Francia et al. 2012). Together, DDRNAs and dilncRNAs promote signalling of DSBs by instigating focal recruitment of DDR proteins downstream of the primary responder γH2AX in the DDR signalling cascade, so-called secondary recruitment. Indeed, localization to the DSB of the DDR mediators MDC1 and 53BP1 is impaired in the absence of DROSHA and DICER, while localization of the DSB sensor NBS1 (Francia et al. 2016), and phosphorylation of H2AX (Francia et al. 2012) are not. When exogenous fluorescent DDRNAs are transfected into a damaged cell they localize to the damaged locus with which they share homology in a transcription-dependent manner, suggesting that DDRNAs can be recruited to the DSB in a sequence-specific manner. Indeed, we found that 53BP1 interacts with DDRNAs (Michelini et al. 2017), and its DSB localization is dependent on dilncRNAs and DDRNAs (Francia et al. 2012, Michelini et al. 2017).
Figure 5. dilncRNAs and DDRNAs are induced at DSBs and promote DDR focus formation

MRN recruits RNA Pol II (RNAP II) to DSBs where it transcribes dilncRNAs, which are then processed by DROSHA and DICER into DDRNAs. Together dilncRNAs and DDRNAs recruit secondary DDR proteins to the DSB in a sequence-specific manner. ASO-mediated inhibition of dilncRNAs/DDRNAs reduces DDR focus formation.

We have also generated evidence that dilncRNAs can function alone at DSBs to promote the DDR through multiple mechanisms. Specifically, we found that following resection of DSBs, BRCA1 is recruited by direct binding of the newly formed DNA-RNA hybrid. Then, by binding to BRCA1, partner and localizer of BRCA2 (PALB2) recruits BRCA2, which in turn brings RNase H2 to clear the RNA-DNA hybrid and allow for the full HR response (D'Alessandro et al. 2018). The discovery of another function of dilncRNA result started with our initial observation that permeabilized cells treated with RNase A display diminished 53BP1 foci (Francia et al. 2012), suggesting that dilncRNAs and/or
DDRNAs may also assist in the maintenance of DDR foci, not only the formation. Indeed, recently we have uncovered a novel role for dilncRNA in inducing phase-separation of 53BP1 at the site of DSBs in order to promote DDR focus formation (Pessina et al. 2019), which is explored in the discussion. Finally, we have also observed DDRNA and dilncRNA induction and function at dysfunctional telomeres, which will be expanded on in section 1.2.

The induction of RNA transcription at DSBs is in seeming contradiction with published observations that RNA Pol II transcription of gene-coding regions is inhibited by a nearby DSB in an ATM-dependent manner (Shanbhag et al. 2010, Pankotai et al. 2012, Iannelli et al. 2017). Messenger RNA (mRNA) transcription was seen in these reports to be reduced by nearby DSBs, while non-coding RNA may escape this silencing. In fact, our group has demonstrated that dilncRNAs extend up to 2kb away from the DSB (unpublished data), while gene silencing at DSBs extends up to 1mb away (Iannelli et al. 2017). Additionally, DSB-induced silencing is ATM-dependent (Iannelli et al. 2017), while dilncRNA induction at DSBs is ATM-independent (Michelini et al. 2017), implying that dilncRNAs and DDRNAs play a role in the DDR upstream from ATM signalling and DSB-induced silencing. Thus, these differences can easily be reconciled by a model in which DSBs cause relaxation of local chromatin, thus allowing RNA Pol II loading and recruitment of DDR proteins, followed by eventual silencing of the region to prevent regular transcription processes from colliding with the DDR focus.

1.2 Telomeres: structure, function, lengthening by telomerase

Telomeres, the very ends of chromosomes, were originally thought to protect the chromosome ends from being fused to one another (McClintock 1941), representing the end-protection problem. Then, with the discovery of the ‘Hayflick limit,’ or the maximum amount of divisions a cell can tolerate, the end-replication problem came into view (Watson 1972). The end-replication problem lies in the inability of cells to fully replicate the lagging strand at the ends of the chromosomes due to the necessity of an RNA primer to start replication on lagging strands that cannot be filled in once removed, thereby leading to the shortening of telomeres with every cell division. To the credit of these researchers, focusing on these aspects of telomeres allowed for substantial advancement of our understanding. Over the years we’ve discovered how intricately telomeres are woven into cellular functions, putting them at the forefront of both cancer and ageing research. The list of mechanisms that we now know cells use to maintain telomeres and to perform telomere functions continues to expand, and indeed many of them are to counteract the end-protection and end-replication...
Despite having gained a vast amount of knowledge about telomeres, there undoubtedly lies just as much, if not more, that we do not yet know.

1.2.1 Structure and function

Telomeres are the nucleoprotein structures found at the ends of all linear chromosomes. In humans and other vertebrates, telomeres consist of hexameric TTAGGG repeats, thus containing a ‘C-rich’ and a ‘G-rich’ strand, which are bound by a specific set of six proteins called the shelterin complex. The six members of shelterin are Telomeric repeat factor 1 and 2 (TRF1/2), which bind to the double-stranded DNA (dsDNA) of telomeres as homodimers (Bianchi et al. 1997, Bianchi et al. 1999), TRF1-interacting nuclear protein 2 (TIN2) that links and stabilizes TRF1 and TRF2 homodimers, TPP1 that links the ssDNA telomere binding protection of telomeres 1 (POT1) protein to TIN2, and finally the TRF2-binding protein RAP1 (Figure 6a).

Telomeres end with ssDNA 3’ G-rich overhangs, which can loop back and invade the upstream telomere sequence creating a telomeric loop, or ‘t-loop’ (Figure 6b) (Griffith et al. 1999). Formation of the t-loop, dependent on TRF2 (Doksani et al. 2013), is thought to solve the end-protection problem by ‘hiding’ the ends of the chromosome. T-loop

![Figure 6. Shelterin and telomeric structure](image)

**Figure 6. Shelterin and telomeric structure**

(a) TRF1 and TRF2 homodimers bind to telomeric dsDNA and are connected to one another and stabilized by TIN2, and TRF2 interacts with RAP1. POT1 binds ssDNA, and is recruited to shelterin by TPP1, which is bound to TIN2. (b) the t-loop structure is formed by TRF2, ssDNA in the d-loop is bound by POT1.
formation by TRF2 seems to occur in two steps: TRF2 first anchors itself to telomeric dsDNA through its MyB/SANT domain (Bianchi et al. 1999), then it wraps ~90bp of dsDNA around its homodimerization TRFH domain (Benarroch-Popivker et al. 2016), thought to promote local unwinding by providing torsional stress thereby allowing for strand invasion. The displaced G-rich telomeric strand forms the displacement loop, or ‘D-loop.’ The ssDNA of D-loops are bound by POT1 (Palm et al. 2009).

Various DDR pathways are inhibited at telomeres, chiefly through the functions of TRF2 and POT1 to inhibit DDR signalling. TRF2 inhibits ATM signalling mainly through t-loop formation by hiding the 3′-end from being recognized by MRN (Celli et al. 2005, Attwooll et al. 2009), which also prevents KU70/80 recognition of chromosome ends. The ‘inhibitor of DDR’ (iDDR) domain of TRF2 prevents ATM-dependent 53BP1 accumulation (Okamoto et al. 2013), and in the context of failed t-loop formation, such as in the case of the TRF2 ‘Top-less’ allele which is defective for t-loop formation due to mutations in its TRFH domain, TRF2-bound RAP1 prevents c-NHEJ despite ongoing ATM signalling (Benarroch-Popivker et al. 2016). Through t-loop formation, TRF2 also inhibits KU70/80 detection of chromosome ends. Similar to how TRF2 inhibits ATM signalling by hiding DNA ends, it is thought that POT1 inhibits ATR signalling (Denchi et al. 2007) by hiding ssDNA from RPA. It has also been suggested that shelterin inhibits telomeric DDR by inducing compaction of telomeric chromatin (Bandaria et al. 2016), however other groups have seen no correlation between telomere compaction and DDR inhibition (Timashev et al. 2017, Vancevska et al. 2017). This discrepancy may be explained by the tendency of dysfunctional telomeres to coalesce (Timashev et al. 2017).

Replication through telomeres is inherently difficult due to the repetitive G-rich strand, which has the propensity to form G-quadruplexes (G4s), and the presence of RNA-DNA hybrids. TRF1 loss results in frequent telomere fork stalling (Martínez et al. 2009, Sfeir et al. 2009), that may be attributed to two distinct functions: as BLM displays G4 unwinding properties (Huber et al. 2002), its TRF2-mediated recruitment assists in telomere replication (Zimmermann et al. 2014); and in conditions of replication stress TRF1 seems to partially inhibit ATR signalling through recruitment of TPP1/POT1 (Zimmermann et al. 2014). The loss of RTEL1 was also linked to increased G4s at the telomere (Vannier et al. 2012), and its loss is epistatic with loss of BLM (Sfeir et al. 2009) suggesting equal functions in resolving G4s.

Following replication of telomeres, the 3′overhang must be formed in order to allow for t-loop formation. While the lagging strand will naturally have a small 3′ overhang due to the end-replication problem, the leading strand will not. The Apollo nuclease, bound to TRF2, first resects the 3′ end of leading strand telomeres thereby generating an appropriate
substrate for Exo1. Further 3’ resection by Exo1 of both leading and lagging strand telomeres generates a large 3’ overhang, which is then filled in to the appropriate overhang length by the CTC1/STN1/TEN1 (CST) complex in combination with Pola/primase (Wu et al. 2012). Following end processing, there is evidence that heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) acts to swap out RPA at the newly generated 3’ overhang for POT1 (Flynn et al. 2011). Following replication and POT1 binding of the 3’ ssDNA overhang, t-loop formation is dependent on TRF2 as described above.

Although t-loops display various properties beneficial to telomeres, they closely resemble a Holliday junction and its processing as such could lead to drastic loss of telomeric material. T-loops can be processed by the endonucleases MUS81 and GEN1, and the resolvase SLX4 (Saint-Leger et al. 2014). poly-ADP-ribose polymerase 1 (PARP1) can recruit Holliday junction resolvases to the telomere (Rai et al. 2016, Schmutz et al. 2017) upon recognition of an available activation site at the end of the 3’ strand, where there is the ds-ssDNA transition. TRF2 counteracts PARP1 loading at telomeres thanks to its N-terminal branched-DNA binding basic domain (Schmutz et al. 2017), likely through physical blocking of the PARP1 activation site. It has also been demonstrated that the basic domain of TRF2 can mitigate branch migration of the t-loop to form a double Holliday junction (Schmutz et al. 2017), a prime target for resolvases. In absence of this basic domain, RAP1 is still sufficient to inhibit recruitment of Holliday junction factors such as the resolvase SLX4 (Rai et al. 2016), and BLM has also been shown to reduce t-loop cleavage (Schmutz et al. 2017), possibly through reversal of the branch migration and restoration of the original t-loop structure (Bizard et al. 2014).

In order to counteract the loss of telomeric material that accompanies each cell division, which may lead to telomere-dysfunction induced senescence (d’Adda di Fagagna et al. 2003), cells activate a telomere maintenance mechanism (TMM). The most well-known and studied TMM is telomerase, responsible for telomere length maintenance throughout generations of mammals and other organisms, which functions by catalyzing the addition of ssDNA G-rich telomeric sequences to the end of the telomere. Its main components are telomerase reverse transcriptase (hTERT) and telomerase RNA component (hTERC), which together are sufficient to induce telomere lengthening (Weinrich et al. 1997). In vivo telomerase occurs as a holoenzyme, that in addition to hTERT and hTERC includes the accessory proteins NHP2, NOP10, Dyskerin, and GAR1. The activity of telomerase is highly regulated, and activated freely only during embryogenesis, then during and after development is restricted to germ cells, stem cells, and activated lymphocytes (Wright et al. 1996). Recruitment to telomeres occurs mainly through TPP1 (Xin et al. 2007, Zhong et al. 2012), and it seems to exhibit a preference for short telomeres (Teixeira et al.
A G-rich telomeric transcript, termed telomeric repeat-containing RNA (TERRA), may also play a role in telomerase recruitment and activity as described below. Following telomerase-mediated extension of the 3’ G-rich end, the complementary C-strand is synthesized in a CST- and DNA polymerase α-dependent manner (Dai et al. 2010).

1.2.2 Telomeric transcription

Following the initial discovery that both human and yeast telomeres are transcribed, resulting in G-rich telomeric repeat-containing RNA (TERRA) with the repeat sequence UUAGGG (Azzalin et al. 2007, Luke et al. 2008), several unique roles for telomeric transcripts, both at and away from the telomere, have been uncovered, along with other telomeric transcript species. TERRA transcription is initiated from within the subtelomere and extends towards the chromosome end, ranging in length from 100bp up to 9kb (Azzalin et al. 2007). TERRA length seems to correlate with telomere length (Arnoult et al. 2012), and a transcriptionally-inducible telomere reveals that TERRA can be extended up to 5-6kb into the telomere sequences (Farnung et al. 2012). Furthermore, rapid amplification of cDNA ends (RACE) to map the 3’ end of TERRA, combined with a northern blot using a subtelomere-specific probe revealed that TERRA can start ~250nt upstream from the telomeric tracts, extending up to 5kb, suggesting that TERRA can extend up to several kb into the telomere (Nergadze et al. 2009). Together, these results suggest that TERRA can be composed of several kb of UUAGGG repeats. Only ~7% of human TERRA species are polyadenylated (Azzalin et al. 2008), but most display a 7-methylguanosine (m7G) cap on their 3’ end (Feuerhahn et al. 2010), thereby promoting stability.

Regulation of TERRA transcription is a multi-faceted process. Indeed, analysis of several human TERRA species revealed promoter binding by multiple transcription factors, including some that repress expression, as well DNA methylation control of TERRA expression at the subset of TERRA promoters that contain CpG-island sequences (Feretzaki et al. 2019). TERRA expression is also inhibited by the telomere-binding proteins TRF1 (Porro et al. 2014a, Sadhukhan et al. 2018, Porreca et al. 2020) and TRF2 (Porro et al. 2014a, Feretzaki et al. 2017). Upon TRF2 loss and telomere uncapping TERRA is upregulated and recruits the H3K9 trimethylation (H3K9me3) histone methyltransferase SUV39H1, thereby enforcing telomeric chromatinization (Porro et al. 2014a). Increasing telomere length seems to negatively regulate telomeric transcription as longer telomeres express longer TERRA molecules thus boosting recruitment of SUV39H1, thereby increasing telomeric H3K9me3 marks, which together with TERRA recruits HP1α, leading to a negative feedback loop resulting in repression of TERRA expression (Arnoult et al. 2012). Consistently, TERRA expression is increased at short telomeres in both Saccharomyces cerevisiae and

Although the roles of TERRA in telomerase modulation have not yet been fully uncovered, it is apparent that the relationship is intricate. *In vitro*, TERRA-like molecules have been shown to inhibit telomerase activity by binding complementarily to its RNA moiety hTERC (Redon et al. 2010). Additionally, ASO-mediated depletion of TERRA resulted in decreased telomerase activity in mouse embryonic stem cells (Chu et al. 2017). However as described above, at short telomeres in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* TERRA is induced and also promotes localization and activity of telomerase (Iglesias et al. 2011, Pfeiffer et al. 2012, Cusanelli et al. 2013, Moravec et al. 2016). Additionally, in human cells telomerase-mediated telomere extension is unaffected by telomere transcription (Farnung et al. 2012). Interaction with hnRNPA1 seems to be important for TERRA-mediated activation of telomerase, specifically the levels of each need to be finely tuned to allow for proper telomere extension: hnRNPA1 can bind to telomeres, TERRA, and telomerase, while TERRA can compete with hnRNPA1 for telomerase binding (Redon et al. 2013). If TERRA levels are low then hnRNPA1 prevents telomerase loading at telomeres, and conversely if TERRA levels are high then telomerase may be bound by TERRA and thereby inhibited, however if TERRA and hnRNPA1 levels are balanced, hnRNPA1 and TERRA bind to one another, allowing for telomerase-mediated extension of telomeres (Redon et al. 2013). Together, these results suggest that TERRA may play a role both in recruitment and activity modulation of telomerase, and that this process is tightly controlled.

TERRA seems to perform multiple functions in safeguarding both telomeric (Bettin et al. 2019). TERRA participates in capping of telomeres by acting together with heterogeneous nuclear ribonucleoproteinA1 (hnRNPA1) to enact an RPA-to-POT1 switch at single-stranded telomeric DNA (Flynn et al. 2011). TERRA may also promote NHEJ of dysfunctional telomeres, as the ATM activator KAT5 is activated by H3K9me3 marks, whose deposition in telomeres following TRF2 loss relies on TERRA-dependent SUV39H1 recruitment (Porro et al. 2014a). Interactions of MRE11 with Lysine-specific demethylase 1 (LSD1) at uncapped telomeres is promoted by TERRA, which in turn allows for processing of the 3’ overhang (Porro et al. 2014b). Interestingly, TRF2 was found to promote RNA-DNA hybrid formation at telomeres by allowing for the strand invasion of TERRA-like RNA into telomeric DNAs, which is prevented by TRF1 (Lee et al. 2018), suggesting that TERRA-based RNA-DNA hybrids at telomeres are controlled in a precise manner. In line with this, cancer cells that maintain their telomere lengths through the
Alternative Lengthening of Telomeres (ALT) pathway, a homology-directed repair-based mechanism, require precise control of telomeric RNA-DNA hybrid levels (Arora et al. 2014).

TERRA roles seem to be at least partially evolutionarily conserved, but human and mouse TERRA stand apart for certain important aspects. As described above, depletion of mouse TERRA seems to boost telomerase activity (Chu et al. 2017), while telomere transcription in human cells does not affect telomerase activity (Farnung et al. 2012). As mouse embryonic stem cells have long telomeres, this discrepancy may be explained by telomere length: TERRA induced at short telomeres (Arnoult et al. 2012) is increasingly polyadenylated in Schizosaccharomyces pombe, which then supports telomerase recruitment (Moravec et al. 2016); TERRA at longer telomeres contains longer UUAGGG tracts (Arnoult et al. 2012), to which SUV39H1 can bind, promoting telomeric H3K9me3 deposition and thus HP1α recruitment (Porro et al. 2014a), which opposes telomerase activity (Chow et al. 2018). Alternatively, increased TERRA levels at long mouse telomeres may inhibit telomerase function by binding to it (Redon et al. 2013). Another possible explanation for these discrepancies could be origin of TERRA: In human cells TERRA colocalizes relatively equally with many telomeres (Azzalin et al. 2007), and has been found to be transcribed from many subtelomeres (Porro et al. 2014a, Feretzaki et al. 2017, Feretzaki et al. 2019), while mouse TERRA aggregates mainly in a few large nuclear foci, and to a lesser extent with few telomeres (Deng et al. 2012, Chu et al. 2017), and may be transcribed from the pseudoautosomal region (PAR) located within Xq/Yq subtelomeres (Chu et al. 2017). It was also proposed that the 18q subtelomere may be the main source for TERRA expression in mouse cells, which then associates with other telomeres in trans (Lopez de Silanes et al. 2014). Thus, it seems that while human TERRA is transcribed from many telomeres, mouse TERRA may be transcribed at non-telomeric regions, or few telomeres. TERRA-dependent recruitment and activation of telomerase could occur in cis, making origin of transcription crucial. Despite unique origins for TERRA expression between human and mouse cells, and a potentially diverse mechanism of control on telomerase activity, in each organism it serves overlapping functions at the telomere, for example TERRA depletion results in increased telomeric DNA damage responses both in mice (Chu et al. 2017) and humans (Avogaro et al. 2018). Determining the origins of TERRA requires further investigation, and together with further elucidation of functions in each organism and will likely shed light on differences between mouse and human TERRA.

Other telomeric non-coding RNAs (tncRNAs) in Schizosaccharomyces pombe have also been described: ARRET, transcribed from within the subtelomere away from the ends; αARRET, transcribed from the subtelomere towards the end, but not reaching telomeric
sequences; and ARIA, a C-rich telomeric RNA transcribed from within the telomere towards the subtelomere, but not reaching the subtelomere (Bah et al. 2012). Interestingly, deletion of Rap1 or Taz1 (homologs of the mammalian proteins RAP1 and TRF1/2, respectively) induced the C-rich ARIA telomeric transcripts (Bah et al. 2012, Greenwood et al. 2012), indicating an evolutionarily conserved increase of tncRNA transcription upon induction of telomere dysfunction.

We have recently characterized tncRNAs also induced at dysfunctional telomeres that are analogous to dilncRNAs and DDRNAs described above in section 1.1.3. We first found that at dysfunctional telomeres, induced by uncapping upon TRF2 loss, telomeric dilncRNAs (tdilncRNAs) are induced, processed by the RNAi machinery DICER and DROSHA into DDRNAs, and together fuel DDR focus formation and maintenance (Rossiello et al. 2017). Specifically, we quantified telomeric DDRNAs (tDDRNAs) through gel-extraction of RNAs under 40bp followed by reverse transcription–quantitative PCR (RT-qPCR), as well as by targeted enrichment of small RNAs (TEsR) (Nguyen et al. 2018), in which a biotinylated bait RNA is used to pull down a target small RNA, followed by sequencing. We found that teloC (C-rich RNAs) and teloG (G-rich RNAs) tDDRNAs are both upregulated 2-3-fold, with the majority of species falling between 19nt and 23nt, consistent with DROSHA product size. In order to quantify tdilncRNAs we used a strand-specific RT-qPCR method, as well as single molecule fluorescence in situ hybridization (smFISH). By RT-qPCR, we uncovered a tdilncRNA induction of ~20 and ~10 fold for teloG and teloC species, respectively, and by smFISH an induction of a ~1.5 and ~20 fold for teloG and teloC species, respectively. Following depletion of either DICER or DROSHA tDDRNA levels dropped, with a corresponding increase in tdilncRNA levels, confirming their status as a tDDRNA precursor, and focus formation of pATM and pS/TQ was impaired. Interestingly, RNase A treatment of permeabilized cells also reduced focus formation, suggesting that telomeric DDR foci maintenance is at least partially RNA-dependent. Finally, we inhibited tncRNA function in a sequence-specific manner with ASOs, resulting in reduced DDR activation both in vitro in cultured cells, as well as in vivo in mice with TRF2-removal induced deprotected telomeres (Rossiello et al. 2017).

1.2.3 Roles in ageing and disease

It has been discovered over the years of studying telomeres that they are intimately linked to health. Aberrant telomere lengthening has been observed in cancer, and telomere shortening in ageing. Cellular senescence, the permanent cessation from cell cycle progression, may be a link between telomere shortening and ageing. Cellular senescence has been linked to ageing and pathological conditions, as well as in normal tissues following
chemotherapy (Collado et al. 2010, Goldman et al. 2013, Kirkland 2016). Progressive telomere shortening with each cell division eventually results in exposed chromosome ends (d'Adda di Fagagna et al. 2003), which trigger a DDR at the telomere, shown to be irreversible (Fumagalli et al. 2012), thus enacting replicative senescence. Interestingly, replicative senescence seems to be induced by a few short telomeres, rather than a short average (Hemann et al. 2001, Herbig et al. 2004). Along these lines, ionizing radiation induces numerous random DSBs throughout the genome, triggering the appropriate DDRs to resolve them. IR-induced damage that happened to occur at telomeres appears to be persistent, and results in a permanent growth arrest, similar to cellular senescence (Fumagalli et al. 2012).

We investigated a potential link between premature ageing, telomeric DDRs, and telomeric transcription and uncovered a role for tncRNAs induced by telomere dysfunction associated with Hutchinson-Gilford Progeria Syndrome (HGPS). HGPS is an extremely rare premature ageing syndrome, affecting 1 in 4-8 million people, in which a mutation in the LMNA gene encoding for lamin A and lamin C results in aberrant splicing, and thus a truncated form of the Lamin A protein called progerin (Hennekam 2006). Among other phenotypes, patients exhibit accelerated telomere shortening (Decker et al. 2009) and elevated rates of telomere dysfunction (Benson et al. 2010). Nuclei of HGPS cells also display aberrant DDRs and chromosomal instability due to deficits in DSB repair and the DDR in general (Liu et al. 2005), and progerin expression causes premature senescence (Cao et al. 2007, Gonzalo et al. 2015). Importantly, telomerase expression in progeric cells partially rescues cell proliferation and DNA damage loads (Kudlow et al. 2008). We found elevated levels of tDDRNAs and tdilncRNAs in cells expressing progerin, and that their ASO-mediated inhibition reduced telomere dysfunction induced foci (TIFs), as well as growth defects and senescence induced by progerin (Aguado et al. 2019). A progeric mouse model, in which progerin is expressed in skin cells expressing keratin-5 (Sagelius et al. 2008), also displayed elevated levels of tncRNAs and TIFs, which were also sensitive to ASOs. These progeric mice eventually display pathological phenotypes, such as increased senescence and reduced proliferation of cells, inflammation and degeneration of skin, as well as a shorter life span. Importantly, ASO-mediated inhibition of tncRNAs alleviated cell growth and cellular senescence defects, as well as ultimately resulted in an increased life span. These results suggest that in the context of a specific pathology, TIFs may themselves be pathological, and as such ASO-mediated inhibition of their signalling may alleviate some symptoms.
1.3 Alternative lengthening of telomeres

Activation of a TMM is necessary for cellular immortalization due to the end replication problem. This TMM-dependence is largely reflected in tumors, as ~85-90% display telomerase activation (Shay et al. 1997), while the remaining 10-15% mostly engage in the alternative lengthening of telomeres (ALT) pathway, but a new class of tumor lacking any TMM has recently been discovered (Dagg et al. 2017, Viceconte et al. 2017). ALT is characterized by telomeric HR that leads to telomere lengthening (Dunham et al. 2000), a conundrum given the wide-ranging ability of telomeres and shelterin to evade DDR activation. There is evidence for evolutionarily conserved ALT activation, as survivors following deletion of the telomerase homolog in Saccharomyces cerevisiae activate one of two TMMs: Type I survivors depends on RAD51 and long tracts of homology, while Type II survivors are RAD51-independent but RAD52-dependent and require shorter homology tracts (Ira et al. 2002). Since the discovery of these immortal human cells lacking telomerase (Bryan et al. 1995) the ALT mechanism has been extensively characterized, with much still to learn, and no targeted treatments available yet.

1.3.1 Common ALT phenotypes

Several phenotypes have been attributed to ALT cells, including: elevated levels of telomeric sister chromatid exchange (T-SCE); the presence of ALT-associated promyelocytic leukemia bodies (APBs); extrachromosomal DNA, especially in the form of C-rich telomeric circles called C-circles; heterogeneous telomere lengths ranging from short to long in a single cell; frequent mutations in the histone chaperone a-thalassemia/mental retardation syndrome X-linked (ATRX), its binding partner death- domain-associated protein (DAXX), or the histone which they deposit together H3.3; and aberrant telomeric DDRs. The study of these characteristics has yielded valuable information about the ALT mechanism.

The most central characteristic to the ALT mechanism is telomeric recombination. ALT cells are characterized by elevated sister chromatid exchange rates specifically at the telomere, called telomeric sister chromatid exchange (T-SCE) (Londono-Vallejo et al. 2004), and there is also evidence for inter-telomere exchange (Dunham et al. 2000). This suggests an underlying presence of telomeric DNA damage, supported by the observation that ALT cells frequently have spontaneous TIFs (Cesare et al. 2009). These spontaneous TIFs do not result in increased telomeric fusions, suggesting that shelterin is still functional. These DDRs at ALT telomeres frequently colocalize with APBs.
Promyelocytic leukemia bodies (PML bodies) are formed by an outer shell made of PML protein, and various unrelated other proteins (Lallemand-Breitenbach et al. 2018). In ALT cells there are frequent colocalizations between telomeric material and PML bodies, known as ALT-associated PML bodies (APBs) (Grobelny et al. 2000). APBs contain multiple telomeres, 2-5 on average, and frequently recruit recombination proteins such as RAD51, RPA, BLM, MRN (Draskovic et al. 2009), RAD50, and BRCA1 (Wu et al. 2003), and as such are thought of as the ‘hub’ at which telomere lengthening occurs in ALT. Indeed, telomere maintenance in ALT requires the APB-localization of the DNA damage sensor MRN (Jiang et al. 2005), and artificial induction of APB formation induced mitotic DNA synthesis (MiDAS), a recently emerging hallmark of ALT (Min et al. 2019). Processing of telomeric recombination intermediates at ALT telomeres seems to result in extrachromosomal DNA.

ALT cells display elevated levels of extrachromosomal telomeric circles (t-circles) (Cesare et al. 2004), as well as many other extrachromosomal telomeric species including branched, linear, and partially single-stranded telomeric DNAs (Nabetani et al. 2009). In particular, a class of extrachromosomal telomeric DNA called C-circles have emerged as a highly ALT-specific marker (Henson et al. 2009), and a simple C-circle assay (CCA) for their quantification was published (Henson et al. 2017). C-circles are defined as telomeric DNA circles containing a continuous C-rich strand, and a discontinuous or gapped G-rich strand. Briefly, the CCA entails a long, single rolling circular amplification step with the Φ29 DNA polymerase. This polymerase displays strand-displacement activity thereby allowing it to endlessly amplify a continuous, circular template, while non-circular templates will be amplified once to the end of the molecule. The products of this C-circle amplification are long, G-rich DNA tails that can be detected with C-rich telomeric probes. Extrachromosomal telomeric DNAs may result from recombination at telomeres, as it has been shown that t-circle generation in ALT is dependent on the HR proteins XRCC3 and NBS1 (Compton et al. 2007). Interestingly, very long telomeres also in non-ALT cells are trimmed, thereby generating t-circles (Pickett et al. 2009).

Heterogenous telomere lengths observed in ALT (Bryan et al. 1995, Bryan et al. 1997) may be due to trimming of very long telomeres as above, but has been observed following HR-mediated repair of telomeric DSBs (Liu et al. 2018). Similarly, the preferential elongation of lagging strand telomeres in ALT was recently discovered (Min et al. 2017), providing another HR-based mechanism by which telomere length heterogeneity is generated. Although it has been demonstrated that short telomeres are recombinogenic (Wang et al. 2005), spontaneous TIFs in ALT do not occur preferentially at short telomeres (Cesare et al. 2009) suggesting that damage, and thus elongation of ALT telomeres occurs
independent from telomere length. This length-independence of TIFs in ALT cells should be verified, however, as short telomeres may evade detection, and damaged telomeres tend to cluster, making their individual visualization more difficult.

ATRX, DAXX, and H3.3 mutations are those related most closely to the ALT pathway (Heaphy et al. 2011, Lovejoy et al. 2012, Schwartzentruber et al. 2012). ATRX and DAXX are histone chaperone partners that deposit the histone variant H3.3 at heterochromatic loci independent from replication, including at telomeres, and thereby prevent replication fork stalling (Lewis et al. 2010, Wong et al. 2010). ATRX has been implicated as a suppressor of ALT, as its re-expression in ATRX-null ALT cell lines reduces ALT phenotypes as well as halts telomere lengthening (Clynes et al. 2015, Napier et al. 2015). Repression of ATRX induces ALT phenotypes in a cell line-specific manner (Brosnan-Cashman et al. 2018), and its loss prior to telomere shortening-driven crisis necessitates ALT activation to escape crisis (Li et al. 2019). However, the mechanism by which ATRX inhibits ALT is unclear. Some studies have found that ATRX can promote sister chromatid cohesion (Ramamoorthy et al. 2015, Lovejoy et al. 2020), suppress RNA-DNA hybrids (Nguyen et al. 2017), or modulate macroH2A1.2 deposition at telomeres during replication stress (Kim et al. 2019). These mechanisms may be all linked with a common cause, or may all be independent thereby implicating a multi-faceted role for ATRX in ALT.

### 1.3.2 ALT telomeres are prone to replication stress

Through elaborate studies of all the ALT phenotypes and how their levels respond to various treatments a common theme has emerged: ALT telomeres are prone to replication stress. For example, APBs form preferentially during and after replication (Grobelny et al. 2000), and as ALT telomeres display long-range directed movement towards other telomeres upon DSB induction (Cho et al. 2014) it may be that telomere clustering in APBs is induced by damage during replication. Consistently, arrest of induced-ALT cells led to a reduction of APBs (O'Sullivan et al. 2014). The replication stress-responding SMARCAL1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1) was also recently shown to resolve replication stress at ALT telomeres (Cox et al. 2016).

There is evidence that telomeric replication stress in ALT cells may be due to altered telomeric heterochromatin. SETDB1-mediated heterochromatin has been shown to promote ALT, inducing transcriptional elongation and telomeric localization of recombination factors (Gauchier et al. 2019). ALT cells display decreased telomere compaction relative to non-ALT cells (Episkopou et al. 2014), which was accompanied by increased TERRA expression. TERRA has been shown to induce replication stress at short telomeres (Graf et
al. 2017), which are frequently present in ALT cells given the prevalence of telomere length heterogeneity. Depletion or over expression of RNase H1 proved to be detrimental for ALT cells, whose telomeres it was specifically recruited to (Arora et al. 2014), suggesting that RNA-DNA hybrids formed by TERRA and induced by altered heterochromatin states may be a source of replication stress that cells must carefully control. The presence of various degenerate sequences in ALT telomeres (Conomos et al. 2012, Lee et al. 2014), especially the TCAGGG variant that seems to be increased specifically in ALT cells, may also increase replication stress by disrupting shelterin binding. Interestingly, it was shown that this TCAGGG variant recruits the nuclear hormone receptor class NR2C/F proteins, which then bridge together other NR2C/F-bound loci, as well other NR2C/F-bound telomeres, thereby leading to telomere insertions within the genome and telomeric recombination (Marzec et al. 2015). These nuclear receptors were also found to recruit ZNF827, a zinc finger protein that in turn recruits the nucleosome remodelling and histone deacetylation (NuRD) complex, thereby inducing deacetylation, further telomere-telomere interaction, and recruitment of HR factors (Conomos et al. 2014). A third, and unique mechanism by which TCAGGG degenerate repeats induce ALT was recently discovered in which interaction between nuclear NR2C/F proteins and Fanconi anemia complementation group D2 (FANCD2) mediates recruitment of the MUS81 endonuclease to induce a telomeric DSB, to which the ALT telomere lengthening polymerase POLD3 was recruited (Xu et al. 2019). These observations provide for the possibility that ALT telomere lengthening proceeds following a damage ‘stimulus’ of replication stress.

1.3.3 Mechanism of lengthening

One of the biggest recent breakthroughs in our understanding of ALT was that telomere lengthening is mediated through break-induced replication (BIR) (Dilley et al. 2016), termed break-induced telomere synthesis (BITS) when induced by an acute telomeric DSB. The authors found that replication factor C (RFC) loads proliferating cell nuclear antigen (PCNA) at damaged telomeres, followed by Pol δ-mediated telomere extension up to 70kb. This DNA synthesis occurred outside of S-phase, reminiscent of MiDAS, and was independent from both RAD52 and RAD51, the latter of which was previously shown to be necessary for long-range movement after telomeric DSB induction (Cho et al. 2014). BIR-mediated ALT telomere synthesis was supported by the finding that the replication of some ALT telomeres is conservative and dependent on the Pol δ subunits PolD3 and PolD4, a characteristic of BIR (Roumelioti et al. 2016).
Adapted from (Sobinoff et al. 2017)

Figure 7. BLM and SLX play opposing roles at ALT telomeres

At Holliday Junctions in which a 3′ end of telomeric ssDNA invades telomeric dsDNA, BLM promotes branch migration and POLD3 recruitment, eventually dissolving the Holliday Junction, resulting in APBs, C-circles, and telomere extension events. If the Holliday Junction is instead resolved by SLX4-SLX1-ERCC4, branch migration is inhibited, resolution is promoted, resulting in T-SCE events in the absence of telomere extension.

How ALT cells trigger telomeric DNA synthesis is an intensive area of investigation. In the absence of exogenous damage stimuli, ALT telomere extension and inter-telomere tag copying is dependent on the BLM-topoisomerase IIIa (TOP3a)-RM11/2 (BTR) dissolvase complex, and is counteracted by the SLX4-SLX1-ERCC4 resolvase complex (Sobinoff et al. 2017) (Figure 7). Interestingly, ALT phenotypes seemed to segregate in this pathway, as T-SCE events were induced upon SLX4 overexpression, but C-circles and APBs were induced by BLM overexpression. This suggests that there is a balance between dissolvase and resolvase activity at ALT telomeres, the former of which induces telomere lengthening, while the latter may be used to tolerate damage.

BLM-dependent telomere extension was first observed to be partially dependent on RAD51 (Sobinoff et al. 2017), whereas BLM-dependent MiDAS induced by artificial APB formation (Min et al. 2019) and telomeric synthesis during G2 (Verma et al. 2019) were dependent on RAD52. Meanwhile BITS was independent from both RAD51 and RAD52 (Dilley et al. 2016). The relative contribution of each of these slightly different forms of
telomeric synthesis to ALT in unperturbed conditions remains unclear, however there is an underlying theme common to them all of Pol δ-dependent BIR-induced telomere synthesis.

### 1.3.4 ALT in cancers

The prevalence of ALT in tumors varies based on the tissue, but most are of mesenchymal origin. ALT is especially prevalent in peripheral nervous system, bone, neuroendocrine, and soft tissue cancers (Dilley et al. 2015). Although no targeted treatments are available in clinics yet, recent years have seen the discovery of multiple ALT-specific features, whose targeting seems to result in ALT-specific cell death.

One of the first identified druggable targets for ALT was ATR (Flynn et al. 2015). The authors showed that ATR inhibition (ATRi) led to reduced levels of ALT phenotypes such as C-circles, APBs, and T-SCE, while increasing telomere loss. ATRi led to cell death specifically in ALT cells, accompanied by a significantly lower IC50 than in non-ALT cells. However, another independent group could not replicate this ALT-specific sensitivity to ATRi (Deeg et al. 2016). Deeg et al. suggest that the conditions used previously may have altered the results of viability assays. The concept of targeting replication stress in ALT seems appropriate, but ATRi may be too broad to reveal ALT-specific effects.

Depletion of the histone acetyltransferases (HATs) General control non-derepressible 5 (GCN5) and P300/CBP-associated factor (PCAF) were recently shown to have opposite effects on ALT cells, resulting in the increase of T-SCE and telomere instability, or decrease of T-SCE, APB levels, and telomere stability, respectively (Jeitany et al. 2017). The pan-HAT inhibitor Anacardic acid (AA) was used to further uncover the importance of HATs in ALT, thus its treatment inhibits both GCN5 and PCAF activity. Treatment of ALT cells with AA phenocopied PCAF knockdown (Bakhos-Douaihy et al. 2019), suggesting that effects of GCN5 depletion were due to a non-HAT function. In addition to decreasing ALT phenotypes, AA treatment led to an ALT-specific reduction in cell viability, and an ALT-specific sensitivity to IR. The exact mechanism by which HAT inhibition specifically targets the ALT mechanism is not clear, but the authors hypothesized that it may be due to downregulation of ALT-associated genes. Although this is promising, the lack of a clear mechanism, and possibility that such wide modification of gene expression may have yet-unknown consequences must be explored to reduce potential toxic side effects.

Testis-specific Y-encoded-like protein 5 (TSPYL5) expression was found to be upregulated ALT cells, and it was identified as a novel APB component (Episkopou et al. 2019). This protein interacts with ubiquitin-specific peptidase 7 (USP7), a deubiquitinating enzyme that also interacts with TPP1. Episkopou et al. found that depletion of TSPYL5 induced cell death specifically in ALT cells due to aberrant USP7-dependent ubiquitination
of POT1 within APBs, resulting in its proteasomal degradation, and thus eventual cell death. This effect was due to sequestering of USP7 by TSPYL5, thereby preventing USP7-dependent ubiquitination of POT1. Targeting the interaction between TSPYL5 and USP7, or TSPYL5 itself, are exciting possibilities for a future ALT-specific treatment.

Fanconi anemia complementation group M (FANCM) was found to resolve replication stress at ALT telomeres (Pan et al. 2017). More recently, it was discovered that depletion of FANCM induced cell death specifically in ALT cells (Silva et al. 2019) through unwinding of R-loops, as well as control of BLM activity. While the role of FANCM in unwinding R-loops was demonstrated in vitro, its effect on BLM is less obvious, and remains to be discovered.

The targeting of FANCM, TSPYL5, and potentially HATs or PCAF thus seem to be possible strategies to allow for the specific targeting of ALT cells. Although there exist many other known mechanisms of ALT telomere lengthening, few have been shown to only play a role in the ALT pathway as those listed here. A common theme underlying recent discoveries of the ALT pathway is the prevalence of telomeric replication stress. Interestingly, similar to ALT cells, non-ALT cells also activate telomeric MiDAS in response to replication stress (Ozer et al. 2018), and BIR in response to telomeric DSBs (Dilley et al. 2016), suggesting that the presence of replication stress, rather than a unique response, may be a key differentiating factor between ALT and non-ALT cells. Thus, targeting telomeric replication stress per se may result in sufficient ALT specificity. Further understanding of these mechanisms will hasten development of targeted treatments.

1.4 Antisense oligonucleotides as a tool, and as a therapy

Antisense oligonucleotides (ASOs) are chemically modified oligonucleotides that inhibit complementary RNA function through watson-crack base pairing. These can be used to inhibit the function of both coding mRNA, as well as non-coding RNA. The chemical modifications that are present in each particular ASO confer certain properties, such as increased binding affinity to target RNAs, or increased resistance to nucleases. Inhibition of RNA function with ASOs can be achieved either by sterically blocking the RNA, thereby preventing it from interacting with other molecules, or by inducing degradation of the RNA. ASOs that act by steric hindrance are called mixmers, while those that induce RNA degradation are called gapmers. Mixmers contain modifications throughout the oligonucleotide which allow it to bind to complementary species with high affinity. Interestingly, mixmers have even been used to induce exon skipping (McClorey et al. 2015). Gapmers, on the other hand, contain a central region of 8-12 DNA oligonucleotides, flanked
by chemically modified nucleotides on either side. When paired with complementary RNAs, this central DNA region will be recognized by RNaseH, thereby inducing degradation of the target (Khvorova et al. 2017).

One of the most common modifications in ASOs is a phosphorothioate backbone (PS), a sulfur group added to the phosphate backbone of oligonucleotides (Figure 8a), which confers increased resistance to nucleases, as well as increased uptake by cells, while possible backbone modification is called peptide nucleic acid (PNA), in which the sugar-phosphate backbone is replaced by a peptide backbone (Figure 8b). Another common modification is locked nucleic acid (LNA), the joining of the 2’ oxygen of an RNA base with the 4’ carbon (Figure 8c), that greatly increases both binding affinity for complementary RNAs, and resistance to nucleases. Similarly, 2’-O-Me modified oligonucleotides contain a methyl group attached to each oxygen moiety on RNA bases (Figure 8d), that increases binding affinity to targets and resistance to nucleases.

ASOs are an appealing alternative to siRNAs which rely on the RNA-induced silencing complex (RISC), that typically functions in the cytoplasm and degrades mRNA. ASOs, however, do not require RISC, and as such can function equally well both in the nucleus, as well as in the cytoplasm. This makes ASOs both useful for studying non-coding RNAs that have nuclear roles, as well as appealing for translational purposes. For example, inhibition of the cancer-associated the long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) reduced metastasis formation in mice (Gutschner et al. 2013). Many ASOs are currently in-use or in clinical trials to treat pathologies, including multiple neurodegenerative diseases (Scoles et al. 2019) and multiple tumor types.
(McClorey et al. 2015). Thus, ASOs represent a powerful tool to study RNA function, as well as an exciting translational method of inhibiting pathological RNAs.

1.5 Hypotheses and Aims

Hypothesis 1: Telomeric non-coding RNAs play a role in the Alternative Lengthening of Telomeres pathway

Aims:
- Compare tncRNA levels between ALT and non-ALT cells
- Investigate role of tncRNAs in maintaining cell viability of ALT and non-ALT cells
- Explore role of tncRNAs in the maintenance of ALT phenotypes

Hypothesis 2: Telomeric non-coding RNAs play a role in the DNA replication stress response

Aims:
- Probe the role of tncRNAs in ALT cells in tolerating endogenous replication stress to maintain cell viability
- Quantify impact of DNA replication stress-induction on tncRNA levels
- Determine effect of tncRNA inhibition on viability of cells experiencing DNA replication stress
2 Materials and Methods
2.1 Cell culture

All cell lines were grown under standard cell culture conditions at 37°C and 5% CO2 levels in the presence of 1% penicillin/streptomycin. All cell lines used tested negative for mycoplasma. To replate and split cells, cells were washed in 1X PBS, then trypsinized for 5-10 minutes in the cell culture incubator, and finally resuspended in fresh media to be replated.

U2OS, HCT116, and G-292 were grown in McCoy’s 5A with glutamax supplemented with 10% fetal bovine serum (FBS); SAOS2 was grown in McCoy’s 5A with glutamax supplemented with 15% FBS; IMR90, SW26, and SW39 were grown in DMEM/M199 4:1 supplemented with 10% FBS, 2 mM L-Glutamine; RPE hTERT was grown in DMEM/F12 supplemented with 10% FBS, 2 mM L-Glutamine, 15 mM HEPES, 0.5% sodium pyruvate; HeLa, MG-63, BJ, WI-38 and WI-38 VA13 were grown in MEM with Glutamax or with 2mM L-gluatmine, supplemented with 10% FBS, 1% sodium pyruvate, and 1% non-essential amino acids; BJ ELR was grown in DMEM/M199 4:1 supplemented with 10% FBS, 2 mM L-Glutamine, 1% sodium pyruvate, and 2.5mM HEPES; BJhTERT was grown in DMEM/M199 4:1 supplemented with 10% FBS, and 2mM L-glutamine, and hTERT expression was selected for with 10µg/ml hygromycin. JFCF-6/T.1J/6B, JFCF-6/T.1J/1.3C, JFCF-6/T.1C, and JFCF-6/T.1D cells were a kind gift from professor Roger Reddel and were grown in MEM with Glutamax or with 2mM L-glutamine, supplemented with 10% FBS, 1% sodium pyruvate, and 1% non-essential amino acids. SI14, SI24, 6C3, and 8G2 cells were a kind gift from professor Anabelle Decottignies, and were grown in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, 1% sodium pyruvate, and 1% non-essential amino acids. U2OS Δ20q TERRA knockout and Control cells were a kind gift from Professor Maria Blasco and were grown in DMEM supplemented with 10% FBS and 2mM L-Glutamine. DR-GFP U2OS cells were a kind gift from professor Philipp Oberdoerffer and were grown in DMEM supplemented with 10% FBS, and 2mM L-glutamine. TLR HEK293 cells were a kind gift from Andrew M. Scharenberg and were grown in DMEM supplemented with 10% FBS, 1% L-glutamine, and selected with 1 µg/ml puromycin.

2.2 RNA extraction

Total RNA from cultured cells was extracted with Maxwell RSC miRNA Kit (Promega) without DNase for DDRNA quantifications, and for dilncRNA quantifications total RNA from cultured cells was extracted with Maxwell RSC Blood and Tissue Kit (Promega), according to the manufacturers protocol.
2.3 Real-time quantitative PCR for DDRNAs

1.5-5 µg of Total RNA was mixed with 10-50pg of synthetic spike-in RNA to be used later as a normalizer, denatured at 65°C for 10 minutes, and fractionated on a 15% polyacrylamide 7 M urea gel in 1X TBE, and RNA species shorter than 40 nucleotides were gel-extracted overnight in extraction buffer (0.3M NaCH₃CO₂ pH 5.5, 100µM EDTA, 0.2% sodium dodecyl sulphate (SDS)) at room temperature (RT). Extracted RNA was phenol-chloroform purified, and precipitated in the presence of 20µg glycogen in 75% ethanol at -80°C for at least two hours. The resulting pellets were resuspended in 25µL water, and half was used for cDNA synthesis with miScript II RT kit (Qiagen) with HiSpec buffer, according to the manufacturer’s instructions. 80µL water was added to each cDNA sample, and 6µL were used per well in triplicate for each PCR target. Real time PCR was performed with QuantiTect SYBR Green PCR Master Mix (Qiagen) with a proprietary miScript Universal Primer in combination with a specific primer, listed below. Each reaction was performed in triplicate. miR-125a was used as a control transcript for normalization for most DDRNA quantifications, apart from HU-induced tDDRNAs in Figure 37 which were normalized on spike-in RNA.

Primer sequences (3′-3′ orientation):

- spike-in Fw: CGAATTCCACAAATTGTATCC
- miR-125a Fw: TCCCTGAGACCCTTTAACCTGTGA
- teloG Fw: TAGGGTTAGGGTTAGGGT
- teloC Fw: CCCTAACCCTAACCCTAA

2.4 Real-time quantitative PCR for dilncRNAs

1µg of total RNA was treated with TURBO DNase (Invitrogen) at 37°C for 1 hour to remove contaminating genomic material, then inactivated by the addition of EDTA to a final concentration of 15mM. RNA was then reverse-transcribed using Superscript First Strand cDNA synthesis kit (Invitrogen) with strand-specific primers (Rv primers in the list below), each normalizer was reverse transcribed in the same reaction mix as the target telomeric RNA. A sample without reverse transcriptase was included for each sample tested to control for genomic contamination. qPCR was performed using SYBR Green I Master Mix (Roche). A volume of cDNA corresponding to at least 8 ng of initial RNA was used for each reaction, performed in triplicate on a Roche LightCycler 480 or 96 detection system. Human ribosomal protein lateral stalk subunit (RPLPO) was used as a control gene for normalization as we have found little variation in its expression between cell lines (data not shown), apart from HU-induced tdilncRNAs in Figure 37 which were normalized on 7SK.
Primer sequences (3’-3’ orientation):
Rplp0 Fw: TTCATTGTGGGAGCAGAC
Rplp0 Rv: CAGCAGTTTCTCCAGAGC
7SK Fw: AGGACCGGTCTTCGGTCAA
7SK Rv: TCATTTGGATGTGTCTGCAGTCT
teloC Fw: CCCTAACCCTAACCCTAA
teloC Rv: TTAGGGTTAGGGTTAGGG
teloG Fw: TTAGGGTTAGGGTTAGGG
teloG Rv: CCCTAACCCTAACCCTAA

2.5 C-circle assay (CCA)

Cells were harvested, pelleted, and frozen for later CCA analysis. Cell pellets were lysed in 50mM KCl, 10mM Tris-HCl pH 8.5, 2mM MgCl₂, 0.5% Tween-20, and 0.5% Nonidet-P40 (NP40). DNA concentration in lysis buffer was quantified with the Qubit dsDNA High Sensitivity Assay (Invitrogen, Q33231). DNA was diluted to 20ng/µL, then re-quantified in duplicate to ensure accurate dilution. 1µL of diluted DNA per sample was used for the C-circle assay, in a mix with final concentrations of 1X Φ29 buffer, 4mM dithiothreitol (DTT), 4µg/ml BSA, 0.1% Tween-20, and 1mM each of dATP, dGTP, dTTP, and dCTP. Samples were incubated with (+Φ) or without (-Φ) 7.5 units of Φ29 DNA polymerase (NEB) for 4 hours at 30°C, then Φ29 was inactivated at 70°C for 20 minutes. Samples were then stored at 4°C until blotting. CCA products were diluted in 2X SSC and dot-blotted onto a positively charged nylon membrane, then cross linked with 1200J of 254nm UV light twice. Cross-linked membranes were then pre-hybridized with hybridization buffer (1.5X SSPE, 10% polyethylene glycol 8000, 7% SDS) for 10-20 minutes at 37°C, probed overnight with 32P-end-labeled telomeric oligonucleotides with the sequence 3’-CCCTAACCCTAACCCTAACC-3’, or with ALU oligonucleotides with the sequence 3’-GTAATCCCAGCCTTTTG-3’. Probes were labelled with T4 polynucleotide kinase (T4 PNK) in 1x T4 PNK buffer (NEB M0201) in the presence of ATP-γ-32P at equal molarity with the oligonucleotides, 8.5µM. Excess 32P was removed from the probes with MicroSpin G-25 Columns (GE Healthcare 27-5325-01), then the probes were eluted in water. Probes were added to hybridization buffer, then added to the membranes for overnight hybridization. Membranes were then washed (washing buffer: 0.5X SSC, 0.1% SDS) three times over 20 minutes, then exposed to a phosphorimager screen. Exposed screens were developed with an Amersham Typhoon Imager (GE Healthcare), and quantified with the associated ImageQuant software. For ALT and non-ALT samples in Figure 10 the
quantification of signal from -Φ samples was subtracted from the quantification of signal from their respective +Φ samples, then these values were divided by 10,000 for the final CCA quantification of each sample. For ASO-treated samples in Figure 29, after developing the telomeric-probed membrane, the membrane was stripped with 0.1% SDS at 65°C until no more radioactive signal is present, about an hour, then re-probed overnight with $^{32}$P-labeled ALU probes. The resulting membrane was then processed, exposed, and quantified as above. Signals for each sample, +Φ and -Φ, was normalized to their own ALU sample. Each of these ratios was then normalized on the Mock +Φ sample, and this ratio was reported.

2.6 Antisense oligonucleotides sequences and chemistries

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Name</th>
<th>Sequence (3’-3’)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNA mixmer</td>
<td>TET (control)</td>
<td>ACTGATAGGGAGTGGTAAACT</td>
<td>Exiqon</td>
</tr>
<tr>
<td></td>
<td>antiteloG</td>
<td>CCCTAACCCTAACCCCTAACCC</td>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td>PS-LNA mixmer</td>
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<tr>
<td></td>
<td>antiteloG</td>
<td>CCCTAACCCTAACCCCTAACCC</td>
<td></td>
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<tr>
<td></td>
<td>antiteloC</td>
<td>GGGTTAGGGTTAGGGTTAGGG</td>
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<tr>
<td>PS-LNA mixmer</td>
<td>Short control</td>
<td>CGTCATAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(scramble)</td>
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<td>Exiqon</td>
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<tr>
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<tr>
<td>PS-2’-O-Me gapmers</td>
<td>Control (LAC)</td>
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<tr>
<td></td>
<td>antiteloC</td>
<td>GGGUUGGGUAGGGGUAGGGGUAGGG</td>
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</tr>
</tbody>
</table>
Table 1 ASOs used for growth curves
ASOs are listed in the order they appear in the text. The phosphorothioate backbone and 2'-O-Me modifications are present throughout each applicable ASO, while LNA modification locations are mixed throughout the ASO, specific locations not provided. Red letters indicate PS-backboned DNA bases, which RNaseH will recognize to induce degradation of target RNAs. For control ASOs the name listed in the text appears first in the table, then in parentheses the sequence target or what it was used for in the respective experiments. *Panagene does not provide sequence of PNAs.

2.7 Antisense oligonucleotide transfections and growth curves in 6-wells and 96-wells
Cells were plated in 6-well plates at 30-40% confluency, or 750 cells per well in a 96 well. 24 hours later ASOs were transfected at the indicated concentration for each experiment with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. All ASOs were boiled at 95°C for 5 minutes and cooled on ice before transfection. For 6-wells, one day prior to relative cell number measurements cells were seeded into a 96-well in triplicate. For experiments in which multiple transfections were performed cells were split one day prior to each transfection to a 6-well at 30-40% confluency as well as to a 96-well at the same confluency, and then one day later cells in the 6-well were re-transfected, and cells in the 96-well were measured for relative cell number. For 96-well transfections, 0.06µL of RNAiMAX was used per well, half of the amount recommended in by the manufacturer. Three days after transfection in 96-wells media was replaced with 10:1 media:resazurin, incubated for one hour in the cell culture incubator, then absorbance measured as below. Value of media alone was subtracted from treated cell values, then normalized on their respective mock treatment average. Relative cell number was monitored with the In Vitro Toxicology Assay Kit, Resazurin based (Sigma-Aldrich), according to the manufacturer’s instructions, outlined below.

2.8 Coulter counter cell counting
Following cell trypsinization, 500µl of cells was diluted in 9.5ml of buffer, and counted on a Coulter Counter. Cell number relative to day 0 was obtained by normalizing cell concentrations on day 0, and multiplying by each splitting ratio.

2.9 Resazurin assay
Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide, Sigma-Aldrich) reduction in metabolically active cells generates resorufin (7-Hydroxy-3H-phenoxazin-3-one), which
absorbs at 570 nm. Cells were incubated with a 10:1 mixture of media:Resazurin for 1-2 hours in a 96-well cell culture plate, then absorbance was measured in a Perkin Elmer EnVision™ 2104 Microplate Reader at 570nm. Absorbance of media alone was subtracted from cell values to obtain a final value for each condition.

2.10 IC50 calculation
Prism software was used to calculate the IC50 reported in Figure 21. Specifically: a non-linear fit analysis of inhibitor concentration vs response, with variable slope and four parameters.

2.11 Real-time quantitative PCR for TERRA
1µg of total RNA was reverse transcribed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). A cDNA volume corresponding to 8 ng of starting RNA was input for each real-time PCR reaction using SYBR Green I Master Mix (Roche) on a Roche LightCycler 480 or 96 detection system, and each reaction was performed in triplicate. Human ribosomal protein lateral stalk subunit P0 (RPLPO) was used as a control transcript for normalization. The following primer sequences were used:

Rplp0 Fw: TTCATTGTGGGAGCAGAC
Rplp0 Rv: CAGCAGTTTCTCCAGAGC
20q-1 Fw: CTGGTGCCAGAGTGGATT
20q-1 Rv: CACCTGTTTCTTGTGTCTGG
20q-2 Fw: ACATGGGCGATACTCAGG
20q-2 Rv: CCCACTACTGTGCCTCAA
20q-3 Fw: GAAGTTGCTGGGTCTATGG
20q-3 Rv: ATGGTGACGACACTGTGG

2.12 Protein immunoblotting
U2OS and SAOS2 protein lysates were collected differently.
U2OS: cells were collected in lysis buffer TEB150 (50mM Hepes, 150mM NaCl, 2mM MgCl2, 5mM EGTA, 0.5% Triton, 10% glycerin), and flash frozen in liquid nitrogen until sample preparation for immunoblotting. To lyse, cells were thawed on ice, spun for 15 minutes at +4°C, 13200 RPM. The protein-containing supernatant was saved, and cell debris pellet trashed. Proteins were quantified with the Bradford assay. 20g proteins were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a 0.45µm
nitrocellulose membrane in transfer buffer (25 mM Tris HCl, 0.2 M Glycine, 20% methanol). Transfer efficiency was controlled for by temporarily staining the membrane with Ponceau, which was then washed away with 1X TBS. Membranes were then blocked in 5% milk in 1X TBS-T (Tween20 0.1%) for 1 hour at RT, then incubated overnight with primary antibodies against either CASPASE-3 (Cell Signaling 9661, rabbit, 1:1000), or PARP-1 (a kind gift from Steve Jackson, The Gurdon Institute, Cambridge, UK, mouse, 1:1000), or incubated for one hour with a primary antibody against tubulin antibody (Sigma T5168, mouse, 1:20000). Membranes were then washed with 1X TBS-T, and incubated with horseradish peroxidase (HRP) conjugated-secondary antibodies (Bio-rad, 1:10000) in 5% milk in 1X TBS-T for 1 hour at RT. After 1X TBS-T washes, membranes were incubated for with enhanced chemiluminescent reaction ECL (GE Healthcare). U2OS membranes were exposed to X-ray film and developed on a Carestream Medical X-ray processor.

SAOS2: cells were collected in laemmli (2% SDS, 10% glycerol, 60 mM Tris HCl pH 6.8). Proteins were quantified with the Lowry assay. 40g proteins were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a 0.45μm nitrocellulose membrane in transfer buffer (25 mM Tris HCl, 0.2 M Glycine, 20% methanol). Transfer efficiency was controlled for by temporarily staining the membrane with Ponceau, which was then washed away with 1X TBS. Membranes were then blocked in 5% milk for two hours, at RT as for PARP-1 or tubulin, or for CASPASE-3 blocked overnight at 4°C, then incubated overnight with primary antibodies against either caspase-3 (Cell Signaling 9661, rabbit, 1:1000), or PARP-1 (Serotec, mouse, 1:1000) in 5% milk, or incubated for 20 minutes with a primary antibody against tubulin antibody (Sigma T5168, mouse, 1:20000) in 5% milk. Membranes were then washed with 1X TBS-T, and incubated with horseradish peroxidase (HRP) conjugated-secondary antibodies (Bio-rad, 1:20000) in 5% milk in 1X TBS-T for 40 minutes at RT. After 1X TBS-T washes, membranes were incubated for with enhanced chemiluminescent reaction ECL (GE Healthcare). Membranes were developed on a Chemidoc imaging system (Bio-Rad)

2.13 Flow cytometry
Caspase-3 cleavage analysis: the medium supernatant was saved and spun down with trypsinized cells, washed once in 1X PBS, fixed in 1% formaldehyde on ice for 20 minutes, washed once in 1% bovine serum albumin (BSA) in 1X PBS, then fixed again in 75% ethanol at 4°C until staining. Fixed cells were then washed, and permeabilized with 0.1% TritonX100 in 1X PBS for 10 minutes at RT, then blocked with 10% goat serum in 1X PBS
for 30 minutes at RT, then stained with Caspase-3 antibody (Cell Signaling 9661, rabbit, diluted 1:50) in 1% BSA in 1X PBS at RT, and subsequently conjugated to the fluorophore FITC (Immunojackson, anti-rabbit FITC, 1:50 dilution) in 1% BSA in 1X PBS, then resuspended in 1X PBS at 4°C until flow cytometry analysis.

Cell cycle analysis: the medium supernatant was saved and spun down with trypsinized cells, fixed in 75% ethanol and stained with PI (Sigma, 50µg/ml) and RnaseA (Sigma, 250µg/ml) solution in 1x PBS overnight prior to analysis.

BrdU and cell cycle analysis: cells were pulsed with 33µM BrdU (Sigma) for 20 minutes, collected by trypsinization, fixed in 75% ethanol, denatured in 2N HCl at RT for 25 minutes, to which 3ml of 0.1M Sodium Borate was added for 2 min at RT, stained with anti-BrdU antibodies (BD, mouse anti-BrdU, 1:5 dilution) in 1% BSA 1X PBS for 1 hour at RT protected from light, and subsequently conjugated to the secondary antibody labelled with FITC (anti-mouse FITC, ImmunoJackson, 1:50 dilution) in 1% BSA 1X PBS, then stained with PI (Sigma, 2.5µg/ml) and RnaseA (Sigma, 250µg/ml) in 1X PBS overnight prior to analysis.

Samples were analyzed on a BD Facs CantoII, using a 488nm laser and 530/30 filter for FITC, and 670nm laser and 585/42 filter for PI. Analysis was done using ModfitLT3.0 software. For caspase positive and BrdU positive cells, at least 500 events were analyzed per sample. For cell cycle at least 8000 events were analyzed per sample.

Fluorescent protein expression analysis (For GFP, mCherry, IFP and BFP fluorophores in DR-GFP and TLR cells): cells were collected by trypsinization, fixed in 1% formaldehyde for 20 minutes on ice, then resuspended in 1X PBS until analysis. Acquisition was performed on an Attune NxT machine, using a 640 nm laser and 720/30 filter for IFP, a 405nm laster and 440/50 filter for BFP, a 561nm laser and 620/15 filter for mCherry, and a 488 laser and 530/30 filter for GFP. Analysis was done using FlowJo_V10 software. At least 10⁴ cells were analysed per sample.

2.14 Senescence-associated β-galactosidase assay (SA-β-gal)

ASO-treated cells were grown in a 6-well cell culture dish on coverslips, fixed in 4% PFA for 10 minutes at RT, then washed with 1X PBS and incubated at 37°C in the absence of CO₂ with SA-β-gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside, 0.5 M phosphate buffer at pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂) over night. Cells were then washed with 1X PBS, fixed again with 4% PFA for 10 minutes at RT, permeabilized with 0.2%
Triton X-100 for 10 minutes at RT, washed once more with 1X PBS, incubated with DAPI for 2 minutes in 1X PBS, washed again with 1X PBS and then mounted with mowiol (Calbiochem).

2.15 Antisense oligonucleotide pull-down
U2OS cells were transfected with 20nM biotinylated ASOs (Exiqon) as above, and 24 hours later cells were harvested and streptavidin-coated dynabeads were used to pulldown biotinylated ASOs for four hours at 4°C. RNA bound to ASOs was released by 5 minutes incubation at 95°C in the presence of 10 mM EDTA, and purified with Maxwell Total RNA Purification Kit (Promega). Spike-in RNA was added following the pulldown, prior to RNA extraction, and was processed with the miScript II RT kit (Qiagen), then its cDNA quantities were quantified alongside telomeric RNA eluted from the pulldown. TeloC RNA was detected by strand specific RTq-PCR, but with the primer listed below for reverse transcription. RNA quantities were normalized on input and spike-in, and then on respective TET ASO controls for each experiment.
Reverse transcription primers for telomeric RNAs (3’-3’)
For teloC RNAs: TAGGGTTAGGGTTAGGGT

2.16 Polyacrylamide gel electrophoresis (PAGE) assay for oligonucleotide structure
Oligonucleotides were annealed in 50 mM Tris-HCl, pH 7.4, 100 mM KCl (5 min 95°C, overnight at room temperature). Experiments with the oligonucleotides (5 µM) were performed on 20% Native SDS-PAGE, 30 mM KCl in TBE, and on 20% SDS-PAGE in denaturing 7M urea conditions. The gels were stained with “stains-all” solution.

2.17 Circular Dichroism
Spectra were obtained on a JASCO J-600 spectropolarimeter, equipped with a thermostated cell holder, with 5 µM oligonucleotide solutions in 50 mM Tris-HCl pH 7.4, 100 mM KCl. The spectra were recorded in 0.5 cm quartz cuvette at 25 °C and 95 °C. The spectra are reported as ellipticity (mdeg) versus wavelength (nm). Each spectrum was recorded three times, smoothed, and the baseline subtracted.
2.18 Ultraviolet (UV) melting analysis

UV melting performed using the JASCO V-750 UV–visible spectrophotometer equipped with a Peltier temperature control system (ETCS-761) (Jasco, JP). 5 µM oligonucleotides were first boiled for 5 min at 95°C then annealed overnight at RT in 100 mM KCl and 50 mM Tris-HCl, pH 7.4. Melting curves were recorded at 290 nm in a 0.5 cm path length quartz cuvette, heating the sample from 20 °C to 95 °C at a rate of 0.5 °C/min. The spectra were analysed with Spectra Manager (Jasco, JP).

2.19 Immunofluorescence

Cells were fixed on glass coverslips with PFA 4% 48 hours after ASO transfection and were stained by immunofluorescence with antibodies raised against PML (Santa Cruz sc-966, mouse, 1:100 dilution) and TRF1 (Santa Cruz sc-6165, rabbit, 1:100 dilution). After incubation with blocking solution (0.5% BSA + 0.2% Gelatin from cold water fish skin (Sigma-Aldrich G7765) in PBS 1X) for 1h at RT, cells were stained with primary antibody for 1 hour at RT in blocking solution, washed and incubated with secondary antibodies for 45 minutes at RT in blocking solution in the dark. Samples were washed with PBS, and Nuclei were stained with DAPI (1 µg/ml), then were mounted in mowiol solution (Calbiochem). Images were acquired using a Leica TCS SP2 AOBS confocal laser microscope and co-localization analysis were performed with a ImageJ-based pipeline.

2.20 Chromosome orientation fluorescence in situ hybridization (COFISH)

Cells were labeled with BrdU:BrdC (3:1, final concentration 7.5:2.5 µM) for 20 hours prior to harvesting, and media was replaced with media containing 0.2 µg/mL colcemid during the last 3 hrs to induce an accumulation of cells in mitosis. Cells were harvested by trypsinization, resuspended with hypotonic 0.03 M Na-Citrate added drop by drop while gently vortexing, then incubated at 37°C for 25 min occasionally inverting the tube. Next, cells were fixed in 3:1 methanol:acetic acid added drop by drop while gently vortexing, then spun down at 900RPM for 5 minutes, and again fixed in 3:1 methanol:acetic acid added drop by drop while gently vortexing, then stored at 4°C. Prior to dropping metaphases on slides, slides were prepared by washing once in methanol, then were left to dry until immediately prior to dropping when they were washed in 45% acetic acid and briefly drained. Prior to dropping, cell suspensions were spun down and resuspended in 3:1 methanol:acetic acid, then spun down again and the supernatant was removed, leaving enough volume to drop, generally ~200-300µL. Slides freshly rinsed and drained from 45% acetic acid were held at
an angle of ~30-45° over a biological waste bin, and 100-150 µL of cells was dropped drop by drop onto glass slides and air dried overnight in the dark. The following steps were all performed in the dark, either in alumin foil-covered coplin jars or in a dark chamber. Slides were rehydrated in 1X PBS, then treated with 0.5mg/mL RNaseA (SigmA) in 1X PBS in a dark humid chamber for 10 minutes at 37°C under coverslips. Slides were then washed in PBS, then 2X SSC twice, then stained with 5µg/mL Hoechst 33258 (Invitrogen, H21491) for 15 minutes, washed 4 times with water, then submerged in 2X SSC in 15cm cell culture dishes and exposed to 365nm UV light for 1 minute (warming up instrument for 2-3 minutes beforehand). Slides are then washed with 1X PBS, quickly rinsed with water, and air dried for 10 minutes prior to incubation with 3000U/mL Exonuclease III (NEB, M0206S) in the buffer provided for 10minutes in a dark humid chamber. Next, slides are washed in 1X PBS then fixed in 4% formaldehyde in 1X PBS for exactly 2 minutes, then washed with PBS prior to an incubation in 200mL water, 200mg pepsin (Sigma P7000), and 168µL 37% HCl for 10 minutes at 37°C. Slides were then washed in PBS, and fixed again in 4% formaldehyde in 1X PBS for exactly 2 minutes, then washed with PBS, and dehydrated in an ethanol series of 70%-90%-100% and air dried for 10 minutes. Dried slides were then hybridized with the Cy3-OO-(CCCTAA)3 telomeric PNA probe (Panagene, F1002-5) for G-rich DNA in hybridizing solution (70% formamide, 0.25% blocking reagent [Roche], 10 mM TrisHCl pH 7.2, 2.14mM MgCl, 0.77mM citric acid, 7.02mM Na2HPO4) for 2 hr at RT. After hybridization, slides were rinsed twice with 70% formamide, 10 mM Tris-HCl pH 7.2, 0.1% BSA, and three times with 1x TBS, 0.08% Tween-20, then dehydrated in ethanol as above. After 10 minutes air drying, the slides were incubated as above with an Alexa647-OO-[TTAGGG]3 PNA probe (PNABio, F1014). Slides were rinsed twice with 70% formamide, 10 mM Tris-HCl pH 7.2, 0.1% BSA, and three times with 1x TBS, 0.08% Tween-20, then were washed once more with 1XTBS, 0.08% Tween-20, with DAPI, then dehydrated in an ethanol series as above. They were allowed to air dry for 10', and then mounted with mowiol (Calbiochem). Pictures were obtained with an Upright Olympus AX70 microscope and a 40X objective. Only one probe was considered for analysis per treatment, as described in the text. Ends of chromosome that have at least one signal were counted as signal positive, and the amount of exchanges was estimated by the amount of chromosome ends with two signals.

2.21 Fluorescence in situ hybridization (FISH)

Prior to harvesting metaphases, and media was replaced with media containing 0.2 µg/mL colcemid for 3 hrs to induce an accumulation of cells in mitosis. Cells were harvested by
trypsinization, resuspended with hypotonic 0.03 M Na-Citrate added drop by drop while gently vortexing, then incubated at 37°C for 25 min occasionally inverting the tube. Next, cells were fixed in 3:1 methanol:acetic acid added drop by drop while gently vortexing, then spun down at 900 RPM for 5 minutes, and again fixed in 3:1 methanol:acetic acid added drop by drop while gently vortexing, then stored at 4°C. Prior to dropping metaphases on slides, slides were prepared by washing once in methanol, then were left to dry until immediately prior to dropping when they were washed in 45% acetic acid and briefly drained. Prior to dropping, cell suspensions were spun down and resuspended in 3:1 methanol:acetic acid, then spun down again and the supernatant was removed, leaving enough volume to drop, generally ~200-300 µL. Slides freshly rinsed and drained from 45% acetic acid were held at an angle of ~30-45° over a biological waste bin, and 100-150 µL of cells was dropped drop by drop onto glass slides, denatured on a humid heat block at 80°C for 1 minute, then air dried overnight in the dark. The following steps were all performed in the dark, either in alumin foil-covered coplin jars or in a dark chamber. Slides were rehydrated in 1X PBS, then treated with 0.5mg/mL RNaseA (Sigma) in 1X PBS in a dark humid chamber for 10 minutes at 37°C under coverslips, then washed with 1X PBS, and dehydrated in an ethanol series of 70%-90%-100% and air dried for 10 minutes. Then, slides were denatured at 80°C with the hybridization solution (70% formamide, 0.25% blocking reagent [Roche], 10 mM TrisHCl pH 7.2, 2.14mM MgCl, 0.77mM citric acid, 7.02mM Na2HPO4) and Alexa647-OO-[TTAGGG]3 PNA probe (PNABio, F1014) for 5 minutes, then hybridized at RT in a dark humid chamber for 2 hours. Slides were rinsed twice with 70% formamide, 10 mM Tris-HCl pH 7.2, 0.1% BSA, and three times with 1x TBS, 0.08% Tween-20, then were washed once more with 1XTBS, 0.08% Tween-20, with DAPI, and finally were dehydrated in an ethanol series as above and mounted. Fragile telomeres were scored if the telomeric signal was elongated, or appeared twice on one end, fused telomeres were scored if there was a telomeric signal between the ends of two chromosomes, and telomere loss was scored at a chromosome end absent telomere signal. Fragile and fused telomeres are reported as the fraction of telomeres displaying the respective phenotype over total telomere-positive ends, while telomere loss is reported as the amount of chromosome ends absent a telomere signal over total chromosome ends.

2.22 Terminal Restriction Fragment (TRF) assay
Cells were resuspended in PBS and mixed 1:1 (v/v) with 2% agarose to obtain 10^6 cells per agarose plug, which was then digested with 1 mg/ml proteinase K in supplied buffer (Sigma) overnight and washed thoroughly three times in TE buffer (10 mM Tris pH 8, 1 mM EDTA).
Proteinase was deactivated upon the addition of 1mM phenylmethylsulfonyl fluoride in 1X TE, and stored at 4°C until ready for electrophoresis. Plugs were then washed in TE, then water, then incubated overnight at 37°C with 50 U MboI and 50 U Alu1 (NEB) in CutSmart 1x buffer. Following digestion, plugs were washed in TE and equilibrated in 0.5 × TBE before loading into a 1% agarose-0.5 × TBE gel. The gel was run for 24 h on a pulsed-field apparatus with the following settings: 6V/cm, run time 24h, angle 120°, initial switch time 5 sec, final switch time 5 sec at 14°C. Pulsed-field gels were used in order to separate large fragments, which standard gel electrophoresis is not capable of. The gel was stained with ethidium bromide and imaged with a ruler to line up the ladder, then depurinated in 0.25M HCl for 30 minutes, denatured in 1.5M NaCl 0.5M NaOH for 30 minutes twice, and neutralized in 3M NaCl, 0.5M Tris-HCl pH 7.0 twice. The gel was then transferred overnight to a positively charged nylon membrane by capillary action in 20X SSC, then crosslinked with 1200J of 254nm UV light. The membrane was then rinsed in water to remove excess 20X SSC, and pre-hybridized with Church mix (0.5M sodium phosphate buffer pH 7.2, 1mM EDTA pH 8.0, 7% SDS, 1% BSA) for 1 hour at 65°C. The membrane was then hybridized overnight in Church mix with a 32P-labelled telomeric probe, as described below. The next morning the hybridization mix was removed, and the membrane was washed with pre-warmed Church wash (40mM sodium phosphate buffer pH 7.2, 1mM EDTA pH 8.0, 1% SDS) three times at 65°C. The membrane was exposed to a phosphorimager screen, which was then developed an Amersham Typhoon imager.

Probe preparation: the pSP73.Sty11x plasmid (addgene plasmid #12401) was digested with EcoRI, resulting in an 800bp TTAGGGn band which was gel-purified out. Roughly 200ng of this extracted band was incubated along with 5ng of primer with the sequence 3′-CCCTAACCCTAACCCTAA-3′ in oligonucleotide labelling buffer with dATP, dGTP, dTTP, and 32P-alpha-dCTP, with klenow polymerase. The labelled probe was cleaned up on a G-50 column, and then mixed with Church Mix for membrane probing.

### 2.23 Telomere shortest length assay (TeSLA)

50ng of genomic DNA was incubated in 1x CutSmart buffer with 2mM ATP, 0.5µl T4 ligase (NEB, M0202M), and Telo1-6 ligation oligos at 10nM each (sequences below), at a total volume of 20µl, overnight at 35°C. Reactions are then inactivated at 65°C for 10 minutes, 0.2µl CviAII (NEB R0640S) is added, along with 8.8µl water and 1µl 10X CutSmart buffer, and incubated for 2 hours at 25°C. Then 0.2µl MseI (NEB, R0525S) 0.1µl Ndel (NEB, R0111S), and 0.2µl BfaI (NEB, R0568S) are added, along with 8.5µl water, and 1µl 10X CutSmart buffer, reaching a total of 40µl, and incubated for another 2 hours at 37 °C. 1µl
rSAP (NEB, M0371S) is then added to the reaction, along with 8µl of water, and 1µl of 10X CutSmart Buffer. 10µl of this reaction is added to 1µl 10X CutSmart buffer, 2µl of 10mM ATP, 0.5µl of the AT adapter (described below), 0.5µl of the TA adapter (described below), 1µl of T4 ligase, and 5µl of water, and incubated at 16°C overnight. Samples are then heat inactivated at 65°C for 10 minutes, and diluted 50X in water. 2µl of DNA from the previous reaction is added to 12.5µl of 2X PCR PreMix H (Epicentre, FS9901K), 0.5µl Adapter (described below), 0.5µl TeSLA TP (described below), 0.5µl FailSafe Polymerase (Epicentre, FS9901K), and 9µl water. This mix is then amplified with following PCR program:

\[ \begin{align*}
94°C & \; 2 \text{ min} \\
60°C & \; 30 \text{ sec} \\
72°C & \; 10-15 \text{ min} \\
72°C & \; 15 \text{ min}
\end{align*} \]

The resulting product is then run on a 0.85% gel, and transferred to a positively charged nylon membrane, fixed by UV crosslinking, then probed with a hypersensitive DIG-labeled telomere probe overnight (lai. et al 2016) at 42 °C. The membrane was then washed with 2× SSC, 0.1% SDS at RT for 15 min, then washed twice more with 0.5× SSC, 0.1% SDS at 55°C for 15 min. Following this, the membrane was washed with DIG wash buffer (1× maleic acid buffer with 0.3% Tween-20) for 5 min, then hybridized with 1X DIG blocking solution for 30 min at RT. After blocking, the membrane was hybridized with anti-DIG antibody (Roche) in 1× blocking solution (1:10,000) for 30 min at RT. The membrane was then washed with DIG buffer twice at RT for 15 minutes each. After washing, telomere signals were detected by incubating with CDP-star for 5 minutes.

**Oligo sequences and preparation**

Oligos for telomeric DNA ligation:

| TeSLA Telo 1 | ACTGGCCACGTGGTTTGTGACCCCTAACC |
| TeSLA Telo 2 | ACTGGCCACGTGGTTTGTGATCGATAACCCT |
| TeSLA Telo 3 | ACTGGCCACGTGGTTTGTGACCTCCTAACC |
| TeSLA Telo 4 | ACTGGCCACGTGGTTTGTGATCGACTAACC |
| TeSLA Telo 5 | ACTGGCCACGTGGTTTGTGATCGAAAACCTA |
| TeSLA Telo 6 | ACTGGCCACGTGGTTTGTGATCGAACCTAA |

Telo 1-6: mix Telo 1, 2, 3, 4, 5, and 6 together to make 10^-2 μM for each
Oligos for Subtelomeric DNA ligation:

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TeSLA ADR1 C3S</td>
<td>GGTACTTTGTAAAGCCTGTC</td>
</tr>
<tr>
<td>TeSLA P22 TA</td>
<td>TAGACAGGCTTACAAAGTAACCATGGTGGAGAATTCTGTCCTTCACGCTACATT</td>
</tr>
<tr>
<td>TeSLAP22 AT</td>
<td>ATGACAGGCTTACAAAGTAACCATGGTGGAGAATTCTGTCCTTCACGCTACATT</td>
</tr>
</tbody>
</table>

- TeSLA ADR1 C3S has C3 spacer at the 3’ end in order to avoid DNA ligation
- TeSLA P22 TA and TeSLA P22 AT have C3 spacers at their 3’ ends to avoid DNA ligation, and phosphorylations at their 3’ ends to facilitate DNA ligation
- pre-anneal TeSLA ADR1 C3S with TeSLA P22 TA and TeSLA ADR1 C3S with TeSLA P22 AT to make stocks for subtelomeric DNA ligation

**Adapter preparation:**
- 40 µM TA adapter: 40 µl TeSLA ADR1 C3S (100 µM) + 40 µl TeSLA p22 TA (100 µM) + 20 µl 5X STE
- 40 µM AT adapter: 40 µl TeSLA ADR1 C3S (100 µM) + 40 µl TeSLA p22 AT (100 µM) + 20 µl 5X STE buffer

1. Heat TA adapter and AT adapter up to 94 - 100°C and gradually cool them down to room temp to make 40 µM stocks.
   5X STE: 50 mM Tris pH 8.0, 250 mM NaCl, 5 mM EDTA

Oligos for TeSLA PCR:

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapter</td>
<td>TGTAGCGTGAAGACGACAGAA</td>
</tr>
<tr>
<td>TeSLA TP</td>
<td>TGGCCACGTGGTTTGGATCGA</td>
</tr>
</tbody>
</table>

2.24 SAOS2 cell cycle arrest and transfection, cell number and Incucyte measurements

For SAOS2 cell cycle arrest and release experiments 150,000 cells were plated in a 6-well dish in serum-containing media, then one hour later gently washed with warm serum-free media, and then incubated in serum-free media until transfection. One day after starvation, cells were transfected with 20nM ASOs as per the transfection protocol, except with serum-free media in place of Optimem. 24 hours after transfection, media was replaced with either serum-free media, or serum-containing media. For cell number measurements 48 hours after release cells were incubated with 10:1 media:resazurin in the 6-well for 2 hours, then 110 µL was transferred to a 96-well plate and read as for other resazurin readings. For the incucyte experiment, one day after transfection, immediately following replacement of media with serum-containing, or serum-free media, the IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (Essen BioScience) was added, and caspase monitored every 3 hours for 48 hours in the IncuCyte® S3 Live-Cell Analysis System (Essen BioScience). This proprietary reagent consists of a DNA-binding GFP dye attached to a DEVD peptide by a DEVD
recognition motif, which is recognized and cleaved by activated Caspase-3/7 in apoptotic cells, allowing the dye to freely bind DNA and fluoresce. The IncuCyte® S3 Live-Cell Analysis System (Essen BioScience) contains a widefield microscope with filters for GFP or RFP fluorescence, and 4x, 10x, and 20x objectives, as well as space for up to six cell culture plates. This system is placed within a standard cell incubator, and image acquisition is fully automated. Image analysis is done with proprietary Incucyte® Software that can be fine-tuned for each individual experiment. The relative number of GFP-labeled nuclei is reported.

2.25 ASO and drug treatments for relative cell number analysis and synergy tests

2000 cells of each cell line were seeded in 50 µL medium in two 96-well plates, with the outer wells filled with PBS. The next day ASOs were boiled at 95°C for 5 minutes, then cooled on ice until treatment. ASOs were added simultaneous to hydroxyurea (HU) or ApHidicolin (APH) in 50 µL to the indicated final concentration, or cells were treated with their vehicles water and Dimethyl sulfoxide (DMSO), respectively. Treatments were each performed in duplicate spread between two plates. Three days later media was replaced with 10:1 media:resazurin, incubated for one hour in the cell culture incubator, then absorbance measured as below. Value of media alone was subtracted from treated cell values, and treated duplicate averages were normalized on their respective mock treatment average. Synergy scores were calculated as previously published (Price et al. 2003).

2.26 Zebrafish strains, housing, injection and sampling

Wildtype zebrafish (Danio rerio) from the AB inbred line were raised and maintained under standard conditions (Westerfield 2000). Embryos were raised at 28.5 °C in E3 medium (4.92 mM NaCl, 0.17 mM KCl, 0.295 mM CaCl2x2H2O, 0.333 mM MgSO4x7H2O). 24 h after fertilization 0.003% 1-phenyl-2-thiourea (PTU; Sigma Aldrich, Taufkirchen, Germany) was added to prevent pigmentation. Zebrafish larvae at 2 days post fertilization were anesthetized with Tricaine mesylate (0.01%, Sigma-Aldrich, Taufkirchen, Germany), and 50-100 SAOS2 cells previously live-stained with Hoechst 33342 (Molecular Probes, OR USA), 1 g/ml for 30 minutes, 1 hour before injection-, suspended in PBS, were injected into the mid-brain ventricle. After cell injection, larvae were kept in E3-PTU medium at 33°C. For confocal live imaging (Leica TCS SPE, Germany) 1 day post implantation (dpi), larvae were anesthetized and embedded into 1% low-melting-agarose (PEQLAB Biotechnologie, Erlangen, Germany), diluted in E3 medium, on microscope slides and imaged. Z-stacks of
confocal images from a dorsal view of the cell implants were overlayed into Max-projections and cell nuclei were counted using ImageJ (NIH, USA). Dead cells are detected by Hoechst concentration (saturated blue, small irregular dots) (Xu et al. 2014). The experiment was repeated four times, for a total of 44 and 45 control- and antiteloC-treated ASO treated fish, respectively.

2.27 DR-GFP and TLR systems

DR-GFP: ASOs (listed below) were transfected with RNAiMAX (Invitrogen) at a total concentration of 20nM simultaneous to IScel expression induction by the addition of 5 µg/ml doxycycline to the cell medium, whereupon the target sequence is cut, and subsequent recombination between the cut site and the downstream mutated GFP cassette results in the correct GFP sequence, and thus functional GFP expression.

TLR: HEK293 TLRsce cells were transfected overnight with a pool of ASOs (A2–A4 and B2–B4) at a total concentration of 50 nM final simultaneous to transfection of the IFP-tagged I-SceI and the BFP- tagged donor GFP plasmids, 0.75µg each, using Lipofectamine 2000 (Invitrogen). IFP-tagged I-SceI, pRRL sEF1a HA.NLS.Sce(opt).T2A.IFP (Addgene plasmid # 31484), and the BFP-tagged GFP donor, pRRL SFFV d20GFP. T2A.mTagBFP Donor (Addgene plasmid # 31485).

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Name</th>
<th>Sequence (3’-3’)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-LNA mixmer</td>
<td><strong>Active ASO group 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>TCGGGGTAGCGGTGAAGCA</td>
<td>Exiqon</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CGCCGTAGTCAGGGGTCGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>GCCAGGGCACGGGAGCTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CCAGGTGGTCAGATGAATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>TCAGCTTGCAGTAGGTATTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>GGATCCACCGGTGGCCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>AGCAAGGGCGAGGAGCTGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>ACCGGGGTGGTGCCCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>GAGCTGGACGGCGACGTAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>TTCAGCGTGTCGGGTAGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Active ASO group 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>TAATACCTACGGGAAGCGTA</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>CTGAAGTTTCATCTGACCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CAAACCTGCGGTCGCCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TCGTACCACCCTGACCTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>TGCTTTAGCGTACCCGGCA</td>
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<td>GAGCTGGACGGCGACGTAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>TTCAGCGTGTCGGGTAGGG</td>
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</tr>
</tbody>
</table>
Table 2. ASOs used for DR-GFP and TLR experiments
1-10 were used for DR-GFP experiments, while 2-4, and 7-9 were used for TLR experiments.

2.28 Statistical analysis
P-values were calculated with Prism 7 software, and values less than 0.05 were considered significant. Unpaired t-tests were used to compare pairs of means. Multiple t-test analysis was used to compare multiple pairs of means. Two-way ANOVA with multiple comparisons was used to compare multiple mean of independent samples.
3 Results
3.1 tncRNAs are upregulated in ALT cells, and teloC species are important for cell viability

3.1.1 ALT cells display elevated levels of tncRNAs

ALT cells are characterized by damaged telomeres (Cesare et al. 2009), and their mechanism of telomere elongation is linked to telomeric DNA damage repair (Dilley et al. 2016). Given the recently-discovered role of tncRNAs in modulating the telomeric DDR (Rossiello et al. 2017), we hypothesized that ALT cells may display elevated levels of tncRNAs. To investigate the possibility that tncRNAs are induced at ALT telomeres, we employed multiple pairs of ALT and non-ALT cell lines. We reasoned that the best cells to compare are those of similar origin, employing or not ALT, in order to minimize the contribution of other factors that may affect their accumulation. To be considered a “pair” here, cells must have a common ancestor. Cell pairs used, and relevant information, are listed in Table 3.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TMM</th>
<th>Common ancestor(s)</th>
<th>Method of immortalization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI14</td>
<td>Telomerase</td>
<td>SW39 (telomerase-positive) and IMRB (ALT)</td>
<td>Fusion between SW39 and IMRB (both SV40T-immortalized from IMR90)</td>
<td>(Episkopou et al. 2014)</td>
</tr>
<tr>
<td>SI24</td>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6C3</td>
<td>Telomerase</td>
<td>JFCF-6 (mortal)</td>
<td>SV40T-immortalized from JFCF-6</td>
<td>(Yeager et al. 1999)</td>
</tr>
<tr>
<td>8G2</td>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JFCF-6/T.1C</td>
<td>Telomerase</td>
<td>JFCF-6 (mortal)</td>
<td></td>
<td>(Lovejoy et al. 2012)</td>
</tr>
<tr>
<td>JFCF-6/T.1D</td>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JFCF-6/T.1J/6B</td>
<td>Telomerase</td>
<td></td>
<td></td>
<td>(Jiang et al. 2009)</td>
</tr>
<tr>
<td>JFCF-6/T.1J/1.3C</td>
<td>ALT</td>
<td></td>
<td></td>
<td>(Lovejoy et al. 2012)</td>
</tr>
<tr>
<td>WI38</td>
<td>None</td>
<td>WI38 (mortal)</td>
<td>None</td>
<td>(Bryan et al. 1997)</td>
</tr>
<tr>
<td>VA13</td>
<td>ALT</td>
<td></td>
<td>SV40T-immortalized from WI38</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. List of paired ALT and non-ALT cell lines tested for tncRNA expression.
Pairs are on consecutive columns highlighted in the same color.

Briefly, all cells were harvested at a similar confluency of around 30-50%, and pairs harvested simultaneously. Cell pellets were flash frozen for processing at later times. For tDDRNA quantification RNA species less than 40bp were isolated for analysis via gel extraction. A commercially-available kit for reverse transcription (RT) and qPCR of short
Figure 9. tncRNAs are upregulated in ALT cells with respect to matched, non-ALT cells
tdilncRNA analysis (left column of graphs), and tDDRNA analysis (right column of graphs). Each separate pair of cells was harvested and RNA processed simultaneously and. (a) SI14 and SI24 cells, n=2 for tdilncRNAs, n=2 for tDDRNAs. (b) 6C3 and 8G2 cells, n=2 for tdilncRNAs, n=2 for tDDRNAs. (c) JFCF-6/T.1J/6B and JFCF-6/T.1J/1.3C ALT, n=1 for tdilncRNAs, n=3 for tDDRNAs. (d) JFCF-6/T.1C and JFCF-6/T.1D ALT, n=1 for tdilncRNAs, n=2 for tDDRNAs. (e) WI38 and VA13 cells, n=1 for tdilncRNAs, n=1 for tDDRNAs. Replicates are independent experiments. Where applicable, error bars represent s.e.m. *p-value<0.05, **p-value<0.005, ***p-value<0.0005.
RNAs, miScript PCR system (Qiagen), was used according to the manufacturer’s instructions, with slight modifications as previously described (Rossiello et al. 2017). For tdilncRNA quantification, total RNA was subjected to DNase treatment, followed by strand-specific RT prior to qPCR. tdilncRNA levels were normalized first on RPLPO, and then on the respective non-ALT line within each pair. tDDRNA levels were normalized first on the housekeeper miR-125a, and then on the respective non-ALT line for each pair. We observed an ALT specific upregulation of teloG and teloC species of tdilncRNAs and tDDRNAs in almost all pairs tested (Figure 9).

![Graph showing relative levels of tdilncRNA](image)

**Figure 10. tdilncRNA levels in non-matched ALT and telomerase+ cell lines**

Cells were all harvested, and RNA processed simultaneously. Error bars represent s.d. of technical triplicates. n=1. Experiments performed in collaboration with Matteo Cabrini.

To extend these observations to other cells, in collaboration with Matteo Cabrini, we employed a set of commonly-used lines including three tumor ALT cell lines (U2OS, SAOS2, G292), two tumor telomerase-positive cell lines (HeLa, MG63), and one hTERT-immortalized normal human skin fibroblast cell line (BJhTERT). As these cells are not paired, tdilncRNA levels are shown relative to RPLPO. Two out of three ALT lines displayed higher levels of both teloG and teloC species of dilncRNAs than the other three telomerase-positive cells (Figure 10).

In addition, I performed a C-circle assay to confirm ALT status of these cell lines. ALT cells displayed drastically higher levels of C-circles than non-ALT cells, confirming
Figure 11. C-circle levels of ALT and non-ALT cells
Cells that are compared directly were harvested at the same time. All cell pellets were processed in the CCA in parallel, and quantified as described in the materials and methods.
the activation or not of the pathway (Figure 11). These observations suggest that tncRNA expression is a novel feature that correlates with ALT status.

3.1.2 ASO-mediated inhibition of teloC tncRNAs reduces cell viability specifically in ALT cells

In order to probe the role of tncRNAs in ALT, we inhibited their function with antisense oligonucleotides (ASOs). ASOs are commonly used to inhibit the function of target RNAs in a sequence-specific manner (Scoles et al. 2019). All ASOs used are listed in the materials and methods (Table 1). We designed ASOs against teloC tncRNAs, called antiteloC, against teloG tncRNAs, called antiteloG, and against unrelated or scrambled sequences as controls.

We first monitored the effect of tncRNA inhibition on the ALT cell line U2OS, and non-ALT lines BJhTERT, and BJ ELR. BJ hTERT cells are mortal BJ fibroblasts immortalized by hTERT infection, while BJ ELR cells are BJ cells transformed by expression of oncogenic Ras, hTERT and the SV40 early region. Cells were mock transfected, or transfected with 200nM ASOs containing locked nucleic acid (LNA) base modifications matching the bacterial TET sequence as a control, or matching telomeric RNAs, on days 0, 3, and 7. Cell number was obtained at days 0, 3, 7 simultaneous to transfections, and a final number obtained on day 10. While both telomerase-positive cell lines were insensitive to control ASOs, and ASOs against tncRNAs only slightly affected BJ ELR cell number (Figure 12a,b), the ALT-positive U2OS cell line displayed a specificity sensitivity to antiteloC ASOs, while antiteloG and control ASOs had no effect (Figure 12c), suggesting that ASO-mediated teloG tncRNA inhibition may not affect ALT cell viability.

The addition of a phosphorothioate backbone (PS) to ASOs increases their stability by affording them further resistance to nucleases (Scoles et al. 2019), thereby potentially allowing for similar or greater ASO efficacy at reduced concentrations. Thus, I next mock transfected U2OS cells, or transfected with 20nM PS-LNA mixmer ASOs against teloG and teloC tncRNAs at days 0, 3, and 7, and counted cells at days 0, 3, and 10. At this concentration of PS-LNA, 10-fold lower than LNA ASOs transfected in Figure 11, U2OS cell number was reduced to almost 0 after 10 days of treatment with antiteloC ASOs, while antiteloG ASOs had no effect (Figure 13).
Figure 12. Inhibition of teloC tncRNAs by LNA ASOs reduces cell number specifically in ALT cells

BJhTERT (a), BJ ELR (b), and U2OS (c) cells were mock transfected, or transfected with the indicated 200nM LNA mixmer ASOs at days 0, 3, and 7. (b) antiteloG and antiteloC counts at day 10 are overlapping. Cells were counted with a Coulter Counter on days 0, 3, 7, and 10, and cell number for each cell line was normalized to day 0. Error bars represent s.e.m. n=3. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005 relative to control – TET.

Figure 13. Inhibition of teloC tncRNAs with PS-LNA ASOs reduces cell number of the ALT cell line U2OS

U2OS cells were mock transfected, or transfected with the indicated mixmer PS-LNA ASOs at 20nM on days 0, 3, and 7. Cells were counted with a Coulter Counter on days 0, 3, and 10, and cell number was normalized to day 0. Error bars represent s.e.m. n=3. ****p<0.00005 relative to mock treatment.

I next transfected PS-LNA ASOs into the mortal fibroblast cell line IMR90, and two of its immortal daughter cell lines telomerase-positive SW39 and ALT-positive SW26. Cells were mock transfected, or transfected with the indicated PS-LNA ASOs at 20nM on days 0, 3, and 7, and relative cell number measured on days 0, 3, 7, and 10 with resazurin, a metabolism-based assay. Although both IMR90 cells displayed a slight, yet equal sensitivity to antiteloG and antiteloC ASOs (Figure 14a), and SW39 cell lines displayed a slight sensitivity to antiteloC ASOs (Figure 14b), the ALT cell line SW26 displayed only
Figure 14. ALT-positive cells are more sensitive to antiteloC PS-LNA ASOs than matched mortal or non-ALT cells

IMR90 (a), SW39 (b), and SW26 (c) cells were transfected with the indicated mixmer PS-LNA ASOs at 20nM on days 0, 3, 7, and 10, and relative cell number was measured with resazurin on days 0, 3, 7, and 10. Error bars represent s.d. of technical triplicates. n=1.

Figure 15. ALT tumor cell lines display increased sensitivity to PS-LNA antiteloC ASOs relative to non-ALT telomerase-positive cell lines

Cells were transfected with the indicated mixmer PS-LNA ASOs at 20nM, and relative cell number was measured three days later. Control ASO here matches the LAC sequence. n=3 for HeLa and SAOS2; n=2 for G-292, HCT116, HeLa, BJhTERT, and MG63. Error bars represent s.e.m. *P<0.05 relative to control ASO. Experiment performed in collaboration with Matteo Formenti.
slight sensitivity to antiteloG ASOs, but to a much greater sensitivity to antiteloC ASOs (Figure 14c).

We further extended our investigation of ALT specific-reduction in cell viability following teloC tncRNA inhibition by testing the ALT tumor cell lines SAOS2 and G-292, as well as the telomerase-positive tumor cell lines HCT116, HeLa, and MG63, and the in vitro immortalized BJhTERT cell line for sensitivity to ASOs. For this experiment, we transfected control, antiteloG, and antiteloC ASOs once, and monitored relative cell number three days later. While both ALT lines were sensitive to teloC tncRNA inhibition (Figure 15a), the telomerase-positive lines were not (Figure 15b).

3.1.3 ALT cells are sensitive to antiteloC ASOs, but not antiteloG ASOs, independent of length and chemistry

I next sought to extend our observations to other lengths and chemistries of ASOs. First, we designed a set of “short” 8-nt mixmer PS-LNA ASOs: six antiteloC ASOs, matching each possible hexameric repeat of telomeres, and an antiteloG and scramble control ASO. Short 8-nt ASOs may be more effective than longer ASOs in vivo due to their small size and thus greater uptake by cells (Obad et al. 2011), and thereby have an appealing translational potential. U2OS cells were transfected with these 8-nt ‘short’ PS-LNA mixmer ASOs, and 21-nt PS-LNA antiteloC ASOs at 20nM on days 0, 3, and 7, and relative cell number was measured with resazurin on days 0, 3, 7, and 10. Short antiteloC-4 and short antiteloC-5 ASOs reduced U2OS cell number by day 10, while antiteloC ASOs reduced cell number almost to 0 prior to day 10 (Figure 16). The reasons that only two short antiteloC ASOs reduce U2OS cell number may be due to G-content and self-annealing capabilities, which are explored in the discussion. Next, I extended these results to the mortal cell line BJ to investigate potential non-specific activity of short antiteloC ASOs that reduce cell number.
Inhibition of teloC tncRNAs with short and long PS-LNA ASOs reduces cell number of the ALT cell line U2OS

U2OS cells were mock transfected, or transfected with the indicated mixmer PS-LNA ASOs at 20nM on days 0, 3, and 7. Cell number was measured with resazurin on days 0, 3, 7, and 10, and normalized on day 0. Error bars represent s.e.m. n=3. ****p<0.00005 relative to Short control on day 10.

in U2OS. Thus, I mock transfected, or transfected short control, short antiteloG, short antiteloC-pool, short antiteloC-4, and antiteloC PS-LNA ASO. Indeed, BJ cells were unaffected by all treatments (Figure 17a), while U2OS cell number was reduced by both short antiteloC-4 and antiteloC PS-LNA ASOs (Figure 17b).

![Figure 17](image17.png)

**Figure 17. Inhibition of teloC tncRNAs with short and long PS-LNA ASOs reduces cell number of the ALT cell line U2OS, but does not affect the mortal cell line BJ**

BJ (a) and U2OS (b) cells were mock transfected, or transfected with the indicated mixmer PS-LNA ASOs at 20nM on days 0, 3, and 7. Cell number was measured with resazurin on days 0, 3, 7, and 10, and normalized on day 0. Error bars represent s.d. of technical triplicates. n=1.

At equal molarity 8-nt ASOs “cover” less of the target RNA than 21-nt ASOs, as there will be an equal amount of individual ASO molecules, but 13 less nucleotides per molecule. We thus reasoned that if shorter ASOs were transfected at higher concentrations, they may evoke responses similar to longer ASOs transfected at lower concentrations. To test this, I transfected U2OS and BJ cells once with short ASOs at 60nM, and longer ASOs at 20nM, and compared effects on viability 3 and 6 days later. While none of the ASOs affected BJ cell number (Figure 18a), Short antiteloC-4 ASOs at 60nM had a similar effect on U2OS cell number as the longer antiteloC ASO at 20nM (Figure 18b). Next, I transfected paired cell lines with short control, antiteloG, and antiteloC-4 PS-LNA ASOs at 60nM, and antiteloG and antiteloC PS-LNA ASOs at 20nM, then monitored consequent cell viability...
three days later. While the mortal cell line WI38 (Figure 19a) was largely unaffected by all ASOs, its daughter ALT cell line VA13 displayed a specific sensitivity to both short and longer antiteloC ASOs (Figure 19b).

**Figure 18.** Short and long antiteloC ASOs at concentrations with comparable ‘coverage’ of their target RNA similarly reduce cell number of the ALT cell line U2OS, but not the mortal cell line BJ

BJ (a) and U2OS (b) cells were mock transfected, or transfected with the indicated mixmer PS-LNA Short ASOs at 60nM, and mixmer PS-LNA antiteloC ASO (21nt) at 20nM once on day 0, and cell number was measured with resazurin on days 0, 3, and 6. Error bars represent s.d. of technical triplicates. n=1.

**Figure 19.** antiteloC ASOs reduce cell number of the ALT cell line VA13, but not its paired mortal cell line WI38

WI38 mortal cells (a) and VA13 ALT cells (b) were transfected with the indicated Short (8nt) and longer (21nt) mixmer PS-LNA mixmer ASOs, at the indicated concentrations. Cell number was measured three days later with resazurin. Error bars represent s.d. of technical triplicates. n=1.

Next, I sought to expand tests of telomeric ASO impact on cell viability to other ASO chemistries in U2OS cells. I first tested whether or not a simple DNA oligonucleotide containing no modifications could affect U2OS cells, and as expected it had no effect (Figure 20a). Next, we added the PS modification to DNA oligonucleotides, in the absence
of LNA modifications, and tested them in U2OS cells for sensitivity. I mock transfected U2OS cells, or transfected with a titration of PS-DNA oligonucleotides against either the TET bacterial sequence as control, or telomeric sequences, and measured relative cell number three days later. PS-DNA oligonucleotides all reduced U2OS cell number at the highest concentration tested, 20nM, and thus are likely to be spuriously toxic regardless of their sequence (Figure 20b). Transfection with peptide nucleic acid (PNA) oligonucleotides at 20nM had no effect (Figure 20c). Finally, I directly compared PS-LNA ASOs at 20nM, to titrations of ASOs containing PS and 2’-O-Me modifications, and found that at 20nM the two ASOs evoke similar responses, and as 2’-O-Me ASOs are titrated up they exert increasing effects, while controls never displayed toxicity (Figure 20d).

Figure 20. Effect on U2OS cell number of various oligonucleotides
Cells were transfected with the indicated oligonucleotides, and relative cell number monitored three days later with resazurin. (a) DNA oligos were transfected at 20nM (b) TET, antiteloG, and antiteloC mixmer PS-DNA ASOs were transfected at 0.2, 2, and 20nM (c) the mixmer PS-LNA ASO and the
PNA ASOs were transfected at 20nM (d) mixmer PS-LNA ASOs were transfected at 20nM, while mixmer PS-2’-O-Me ASOs were transfected at 20, 100, and 200nM. Error bars represent s.d. of technical triplicates. n=1.

To more rigorously and quantitatively test ASOs of different chemistries, I performed a high through-put growth analysis of U2OS cells treated with titrations of three different ASO chemistries: PS-LNA mixmer ASOs, called antiteloG and antiteloC thus far; PS-2’-O-Me mixmer ASOs, those used in figure 19; and PS-2’-O-Me gapmer ASOs, which function by inducing RNaseH-mediated degradation. Cells were transfected in duplicate spread across two 96-well plates, and relative cell number was measured three days later with resazurin. While control and antiteloG PS-LNA mixmers (Figure 21a), PS-2’-O-Me mixmer ASOs (Figure 21b), and PS-2’-O-Me gapmer ASOs (Figure 21c) had no effect at any concentration relative to mock transfection, antiteloC ASOs of each chemistry induced a dose-dependent reduction in cell number (Figure 21). At their highest concentration, all antiteloC ASOs drastically reduced U2OS cell number. To compare these three different ASO chemistries, I calculated the concentration at which each antiteloC ASO reduces U2OS cell number by 50%, the IC50, to quantify differential effects. PS-2’-O-Me ASO gapmers displayed the lowest IC50 of ~4.4nM, followed by PS-2’-O-Me ASO mixmers at ~11.5nM, and PS-LNA mixmers at ~21.3nM (Figure 20d).

![Figure 21](image)

**Figure 21.** Inhibition of teloC tncRNAs in the ALT-positive U2OS cell line with multiple ASO chemistries results in a dose-dependent reduction of cell number

U2OS cells were seeded in a 96-well cell culture plate, transfected with ASOs the following day, and three days later relative cell number was measured with resazurin. ASOs were titrated down in steps of 2 from a
maximum concentration of (a) PS-LNA mixmer ASOs at 320nM, (b) mixmer PS-2’-O-Me at 160nM, and (c) Gapmers at 160nM. (d) IC50 was calculated as detailed in the materials and methods.

In conclusion, ALT cells are sensitive to antiteloC ASOs (Figures 12-21) regardless of ASO chemistry (Figures 20,21) and length (Figures 16-19), while non-ALT cells are largely insensitive to antiteloC ASOs (Figures 15, 17, 18, 19) or seem to be spuriously sensitive to ASOs following multiple treatments, regardless of their sequence (Figures 12, 14). While almost all antiteloC ASO chemistries reduced ALT cell number, it seems that PS-2’-O-Me ASOs were the most effective, and gapmers more so than mixmers (Figure 21b,c). While antiteloC ASOs always reduced ALT cell number, interestingly, none of the tested antiteloG ASOs impacted ALT cell number (Figures 12-21), even at very high concentrations (Figure 21). Below I explore explanations for this difference.

3.1.4 Cells with reduced TERRA expression do not display enhanced sensitivity to antiteloG ASOs

Given the apparent relatively equal contribution to telomeric DDRs of both teloG and teloC tncRNA species at uncapped telomeres (Rossiello et al. 2017), and prominent roles for DDRNAs and dilncRNAs coming from both sense and antisense strands surrounding genomic DSBs (Francia et al. 2012, Michelini et al. 2017), it is unexpected that antiteloG and antiteloC ASOs evoke different responses in ALT cells. One potential explanation is that antiteloG ASOs are “sponged” by TERRA in ALT cells, or that the long UUAGGG tracts in TERRA molecules will bind to antiteloG ASOs, depleting them from the nucleoplasm, and thereby preventing them from acting on dilncRNAs. To investigate this possibility, we sought an ALT cell line displaying reduced TERRA expression. We thus obtained a U2OS cell line that display reduced TERRA expression thanks to CRISPR-Cas9-mediated removal of TERRA promoters on the 20q subtelomere (Montero et al. 2016). The conclusion that the 20q subtelomere is a main driver for TERRA expression in human cells is under debate (Feretzaki et al. 2019), and is not investigated here. Instead, here these cells were used to compare response to ASOs of isogenic cells differing for total TERRA levels. Expression of the 20q locus, as well as total teloG tncRNA content were analysed by RT-qPCR, in collaboration with Julio Aguado Perez, in order to confirm reduced TERRA expression relative to the control U2OS line. We, indeed, confirmed the nearly complete ablation of TERRA from the 20q locus, using three separate primers, and a nearly 80% reduction of total teloG tncRNA content in the Δ20q line in comparison with the control line (Figure 22a). Control and Δ20q lines were treated with ASOs, and cell number monitored three days later. While both cell lines were sensitive to teloC RNA inhibition, neither was sensitive to teloG tncRNA inhibition (Figure 22b), suggesting that teloC tncRNAs may play
a role distinct role from teloG tncRNAs at ALT telomeres, differently than at uncapped and dysfunctional telomeres where the two species seem to be equally important in DDR formation and maintenance.

Figure 22. TERRA Δ20q U2OS cells do not display increased sensitivity to antiteloG ASOs
(a) U2OS control and Δ20q cells were harvested together, and total RNA was extracted for telomeric RNA quantification. Error bars represent s.d. of a technical triplicate. n=1. (b) U2OS control and Δ20q cells were transfected with PS-LNA mixmer ASOs at 20nM and cell number was measured three days later with resazurin. Error bars represent s.e.m. n=3. *p<0.05, **p<0.005. qPCRs performed by Julio Aguado Perez.

3.1.5 teloC tncRNA inhibition results in apoptosis, not cellular senescence, in ALT cells
We have shown that treatment with ASOs targeting teloC tncRNAs results in reduced cell number specifically in ALT cells, so we next sought to determine the fate of these ASO-treated cells. Given the roles of DDRNAs and dilncRNAs in both telomeric DNA damage signalling as well as repair (Rossiello et al. 2017), and the important role for DNA damage at telomeres in ALT cells (Sobinoff et al. 2017), we investigated potential cell fates following persistent DNA damage. We monitored activation of senescence and apoptosis, potential outcomes of unrepaired DNA damage (d'Adda di Fagagna 2008), in ALT cells after ASO treatment. Cells were transfected with ASOs at 20nM and analysed two days later for apoptosis and senescence activation, one day prior to all cell number analysis described above. Following activation of apoptosis several proteins are cleaved, such as CASPASE-3 and PARP-1, whose staining allows for the quantification of apoptotic cell number. Performed in collaboration with Francesca Rossiello, treatment of U2OS and SAOS2 cells with antiteloC ASOs induced cleavage of CASPASE-3 as well as PARP-1, as shown by western blot analysis, while mock, control ASO, and antiteloG ASO treatments left cells unaffected (Figure 23a). I also checked for apoptosis activation in U2OS cells by flow
cytometry by monitoring cells positive for cleaved CASPASE-3. Indeed, inhibition of teloC tncRNAs caused a significant increase in the fraction of cells undergoing apoptosis from ~2.5% following mock, control ASO, and antiteloG ASO treatment, to ~11% following antiteloC ASO treatment (Figure 23b). As senescence and apoptosis are both possible outcomes of unrepaired DNA damage, in collaboration with Francesca Rossiello we next monitored senescence activation following ASO treatment. Senescent cells express senescence-associated-β-galactosidase (SA-β-gal), a lysosomal β-galactosidase whose activity can be measured by histochemical staining (Dimri et al. 1995). No ASO-treated U2OS cells were positive for SA-β-gal following Treatments, and a low amount of SA-β-gal positive SAOS2 cells was induced independent from ASO sequence (Figure 23c).

Figure 23. teloC tncRNA inhibition induces apoptosis, not cellular senescence, in U2OS and SAOS2 ALT cells
U2OS and SAOS2 cells were transfected with 20nM PS-LNA ASOs, and harvested two days later for analysis. (a) protein lysates were analysed by western blot. n=1. (b) cells were stained for cleaved-CASPASE-3, and analysed by flow cytometry. n=2 for mock, antiteloG, and antiteloC, n=1 for control. Error bars represent s.d. (c) cells were fixed, and stained for β-galactosidase. At least 200 cells were scored per condition between three independent experiments. Error bars represent 95% confidence intervals of each condition.
performed by Francesca Rossiello, and flow cytometry acquisition performed by the flow cytometry unit in IFOM.

### 3.1.6 antiteloC ASOs bind teloC tncRNA

In collaboration with my colleague Matteo Formenti, we next checked antiteloC ASO binding of target C-rich tncRNAs by ASO pull down followed by RT-qPCR. Biotinylated ASOs were transfected into U2OS cells at 20nM, one day later pulldown with streptavidin-coated dynabeads was performed on cell lysates. Following pulldown, RNA was purified and spike-in RNA was added as a normalizer. The amount of C-rich tdilncRNAs pulled down by biotinylated TET ASOs (control) or biotinylated antiteloC ASOs relative to spike-in RNA was quantified. Indeed, antiteloC ASOs pull down significantly more teloC tdilncRNAs than control ASOs, confirming that antiteloC ASOs bind C-rich tncRNAs (Figure 24).

**Figure 24. antiteloC ASOs bind teloC tncRNAs**

Biotinylated control (TET sequence) or antiteloC ASOs were transfected at 20nM in U2OS cells, and cell lysates were collected 24 hours later for pulldown, and RT-qPCR. n=3. Error bars represent s.e.m. **p<0.05. Experiment performed by Matteo Formenti."
3.1.7 *G*-quadruplex formation of antiteloC ASOs does not explain impact on cell number

It was previously discovered that G-rich oligonucleotides exert anti-cancer properties of (early studies and mechanism reviewed in (Bates et al. 2009), incites to mechanisms, clinical studies, and new uses reviewed in (Bates et al. 2017)). In brief, it was proposed that G-rich oligonucleotides capable of forming G4s exert anti-proliferative effects on cancer cells. These oligonucleotides exert their anti-cancer properties by acting as aptamers and binding to the cell surface protein nucleolin. Importantly, almost all of these studies were done on DNA oligonucleotides, rather than ASOs, cells were treated with micromolar range oligonucleotides in lieu of using a transfection reagent, and no ALT-specificity was uncovered, while DNA oligonucleotides tested here do not reduce ALT cell number (Figure 20a), and antiteloC ASOs do not significantly reduce telomerase-positive cell number (Figures 12, 14, 15, 17, 18, 19).

![Figure 25. ASO running properties on denaturing and native SDS-PAGE](image)

The indicated ASOs (PS-LNA ASO chemistry first four lanes, 2'-O-Me ASO chemistry last lane) and DNA markers were run on a 20% SDS-PAGE at 5μM in 50 mM Tris-HCl, pH 7.4, 100 mM KCl under (a) denaturing 7M urea or (b) native conditions. Lanes for both (a) and (b) were digitally stitched together each from single denaturing, or native gels, respectively, for uncropped gels see appendix. Experiments performed by the group of Professor Xodo.

Nonetheless, to investigate the possibility that the effects on cell viability of antiteloC ASOs are due to G-quadruplex formation, we collaborated with Professor Luigi E. Xodo.
and his research group at the Università degli studi di Udine. Xodo and colleagues ran selected oligonucleotides on a denaturing PAGE gel to assure that they run at their expected length (Figure 25a), and on a native PAGE gel to see if they form secondary structures affecting their electrophoretic properties (Figure 25b). As expected, the PS-LNA mixmer ASOs control and antiteloG run as single molecules in both denaturing (Figure 25a, left two ASO lanes) and native gels (Figure 25b, left two lanes). ASOs with antiteloC sequence, on the other hand, all run as single molecules near the expected length in denaturing gels, but display diverse running properties in native gels. The short antiteloC-4 ASO, that is 8-nt long, may have been run off the gel, unfortunately (Figure 25b, third ASO lane), however if it were to make secondary structures, they likely would have remained on the gel. Meanwhile, the PS-LNA antiteloC and 2’-O-Me antiteloC ASOs do indeed form complex structures (Figure 25b, fourth and fifth ASO lanes, respectively).

Next, to investigate what type, if any, of G-quadruplex antiteloC ASOs are capable of forming, circular dichroism (CD) assays were performed. G-quadruplexes can be made of parallel (oriented in the same 3′-3′ direction), or antiparallel guanosines. In CD, parallel G-quadruplexes absorb around 265nm, whereas antiparallel G-quadruplexes absorb around 290nm. Absorbance was read at 25°C to permit G-quadruplex formation, and at 95°C when G-quadruplexes should be denatured. No structure could be appreciated from control, antiteloG, and short antiteloC-4 ASOs (Figure 26a,b). Although the structure of the short antiteloC-4 ASO could not be appreciated on the denaturing PAGE gel of Figure 25b, by

![Figure 26](image-url).

Figure 26. Circular dichroism study of ASOs for G-quadruplex forming capabilities
CD spectra of indicated ASOS, (a,b,c,d) PS-LNA mixmer ASO chemistry, (e) PS-2’-O-Me mixmer ASO, in 50 mM Tris-HCl pH 7.4, 100 mM KCl. Ellipticity expressed in millidegrees (“CD (mdeg)”), measured at 25°C (blue) and 95°C (red). For melting curves, ASOs were heated from 20°C to 95°C at a rate of 0.5°C/min, and absorbance recorded at 290nm and 260nm. Experiments performed by the group of Professor Xodo.
CD this ASO displayed no structure, suggesting that it does not form G-quadruplexes (Figure 26c). Instead the PS-LNA antiteloc ASOs formed both parallel, and antiparallel G-quadruplexes (Figure 26d). Although the 2’-O-Me antiteloc ASO did absorb around 260nm, it likely did not form G-quadruplexes, as no structure can be appreciated in the melting curve at either wavelength (Figure 26e).

In summary, the PS-LNA antiteloc ASO is indeed capable of forming G-quadruplexes, while two other ASOs, the PS-LNA short antiteloc-4 and 2’-O-Me antiteloc ASOs, do not form G-quadruplexes. Decreased cell number following ASO transfection in U2OS cells is thus unlikely to be mediated by G-quadruplex formation, as both short antiteloc-4 and 2’-O-Me antiteloc ASOs decrease cell number, but do not form G-quadruplexes.

3.2 The effect of ASO-mediated tncRNA inhibition on ALT phenotypes and telomeres

3.2.1 Inhibition of teloC tncRNAs increases the number of cells with many APBs and T-SCE events

To better understand how a treatment affects the ALT pathway, the effect on various ALT phenotypes can be measured. With my colleague Francesca Rossiello we first measured APB levels following ASO treatment in U2OS cells. Cells were fixed on coverslips for APB staining, defined here as the colocalization of PML foci with TRF2 foci, two days after ASO transfections. Results are plotted as the fraction of cells within each treatment displaying a specified range of APB numbers per cell. Antiteloc ASO treatment resulted in an increase in the fraction of cells with few (0-2 bin) APBs, at the cost of cells with many APBs (>12 bin) (Figure 27).

Next, I measured T-SCE events two days after ASO transfections in U2OS cells. The chromosome orientation fluorescence in situ hybridization (CO-FISH) technique, used to quantify T-SCE events on metaphase spreads, allows for the selective degradation of nascent DNA, thus exposing opposite strands of DNA on each sister chromatid. Then, telomeres are stained with fluorescent PNA probes that recognize either teloG or teloC telomeric DNA. A normal pair of sister chromatid telomeres that did not undergo exchange will have one sister stained by one probe, while those that underwent exchange will have both sisters stained by the same probe. As telomeric ASOs have the potential to bind directly to telomeric PNA probes and interfere with their telomere staining (for example Figure 27a, bottom-middle panel reveals weak telomere staining and aggregation of the Cy3-OO-(CCCTAA)3 probe in
antiteloC treated cells), I quantified the effects on T-SCE levels of each telomeric ASO alongside mock and control treatments in separate experiments, matching PNA probes

sequence with ASO sequence to prevent any interaction (i.e. antiteloG ASO treatments were analysed with teloC probes, and antiteloC ASO treatments with teloG probes). T-SCE events per cell were recorded as the fraction of T-SCE-positive ends per metaphase, and reported here as the fraction of metaphases displaying a specified range of T-SCE events. We observed that transfection with antiteloC ASOs results in an increased number of T-SCE events, as indicated by an increase in cells with many T-SCEs (0.2-0.4, 0.4-0.6 bins), mirrored by a decrease in the number of cells with few T-SCEs (0.0-0.2 bin) (Figure 28c). A smaller increase in T-SCE levels relative to mock and control treatments not reaching statistical significance was observed following antiteloG treatment (Figure 28d), however an analysis with more cells should be performed.

Figure 27. antiteloC ASOs decrease APBs in U2OS ALT cells
Cells were transfected with the indicated PS-LNA ASOs at 20nM, fixed two days later, and stained for APBs. (a) Examples of APB immunostaining for TRF2 (red), PML (green). Examples of APBs indicated by white arrows. (b) Quantification of experiments in (a). Each bin represents the fraction of all cells analyzed displaying that range of APBs. At least 60 cells were scored per condition among three experiments. Error bars represent 95% confidence intervals. Experiment performed by Francesca Rossiello.
Figure 28. Effect of tncRNA inhibition on T-SCE events in U2OS cells

U2OS cells were transfected with PS-LNA mixmer ASOs at 20nM, and metaphases were harvested two days later for CO-FISH analysis. (a) Examples of ASO-treated metaphases stained with Alexa647-OO-[TTAGGG]₃ (left), Cy3-OO-(CCCTAA)₃ (middle), merged with DAPI (right). (b) Examples of T-SCE (yellow arrows) and non-exchanged sister chromatid telomeres (white arrows).
3.2.2 The effect of tncRNA inhibition on C-circles

The presence of C-circles is considered the best ALT-specific marker currently available, they can be quantified with the C-circle assay (CCA) and their level correlates with ALT activity (Henson et al. 2009). Telomeric ASOs may interfere with amplification steps within the CCA, or detection of amplification products, as with the CO-FISH assay. I treated U2OS cells with ASOs and monitored C-circle levels two days later. While the control ASO had no effect on C-circle levels relative to mock treatment, it seems that antiteloG ASOs may trigger a decrease in C-circles, while antiteloC ASOs may trigger an increase (Figure 29). antiteloC ASOs seem to be directly detected by the CCA probe, as the signal of the -Φ fraction of the antiteloC ASO-treated sample was comparable to that of the +Φ samples of mock and control treatments. Presumably this signal increase is due to the ASO itself being detected. If this non-specific signal of the ASO can be considered background, then the antiteloC ASO seems to result in a ~2.5-fold increase in CCA signal relative to mock and control ASO. On the other hand, antiteloG ASO treatment seems to reduce C-circle levels, however this ASO may be binding the long G-rich CCA amplification product, thereby preventing it from being detected by the probe. The contribution of these artefacts is not quantifiable in this experiment.

3.2.3 tncRNA inhibition has no impact on telomere fragility, fusion, loss, or length

A potential outcome of DDR inhibition at ALT telomeres, and a possible trigger for induction of apoptosis, could be a sudden induction of fragile or fused telomeres, loss of telomeres, or change in telomere length. To
Figure 30. Effect of tncRNA inhibition on telomere fragility, fusions, and loss in U2OS cells

U2OS cells were transfected with PS-LNA mixmer ASOs at 20nM, and metaphases were harvested two days later for FISH analysis. Telomeres were stained with teloG probes, chromosomes with DAPI. (a) Examples of ASO-treated metaphases. (b) Examples of a fragile telomere (white arrow), telomere loss (yellow arrow), and telomere fusions (red arrow). (c-e) quantifications of (c) fragile,
(d) fused, or (e) lost telomeres. n=2 independent experiments, at least 25 metaphases per condition. Error bars represent 95% confidence intervals.

To monitor fragility, fusion, and loss of telomeres I used fluorescence in situ hybridization (FISH) to label telomeres on metaphase spreads with PNA probes, and scored telomeric phenotypes following ASO treatment. A fragile telomere is visualized in FISH as a double signal, or extended and smeared signal, while fused telomeres appear as two sister chromatids fused at their ends, and telomere loss is marked by a chromosome end void of telomere FISH signals. I treated U2OS cells with ASOs, and analysed telomeres by metaphase FISH two days later. Inhibition of both teloC and teloG tncRNAs with ASOs did not affect telomere fragility, fusion, or loss (Figure 30c,d,e, respectively).

To check for changes in telomere length upon ASO treatment, I first employed the terminal restriction fragment (TRF) assay. I transfected U2OS cells with ASOs, and two days later fixed cells in agarose plugs. Agarose plugs were then digested with a cocktail of MboI and AluI (NEB) endonucleases, and ran on a pulsed-field gel. The gel was stained with Ethidium Bromide (Figure 31a), and then transferred to a nylon membrane. The membrane was then probed with a radio-labeled telomeric probe. Inhibition of tncRNAs had no effect on telomere length (Figure 31b).

The TRF is useful for measuring average telomere lengths and monitoring telomere length distributions, but does not retain information of very short telomeres. Indeed, if antiteloC ASO-mediated inhibition of teloC tncRNAs causes the drastic loss of few telomeres per cell, this information may not be appreciable either through TRF, or FISH. Recently, a new technique called TeSLA was developed by Professor Jerry Shay and his group for this reason: to unbiasedly detect both short and long telomeres (Lai et al. 2017). Briefly, this technique allows for the PCR amplification of individual telomeres ranging from <1 kb up to 18kb, without introducing biases for length. We collaborated with the Shay lab to analyse the fate of telomeres by TeSLA in ASO-treated U2OS cells two days after...
transfection. Additionally, we treated HeLa cells with ASOs and monitored outcomes on telomere lengths as negative control as ASOs have no effect on HeLa cell viability (Figure 15), and thus it is unlikely that telomeres would be affected. ASO transfections did not result in major changes in average telomere length, nor in the length of the shortest 20% of telomeres in both U2OS and HeLa cells (Figure 32a,b).

Importantly, as teloC tncRNAs inhibition induces acute cell death, affected cells may die prior to mitosis. Thus, effects on telomere phenotypes may be hidden due to elimination of affected cells from the population. This is explored in the next section.

![Figure 32. TeSLA analysis of telomere length following tncRNA inhibition in U2OS and HeLa cells](image)

(a) HeLa and (b) U2OS cells were transfected with PS-LNA mixmer ASOs at 20nM, two days later cell pellets were harvested for telomere length analysis by TeSLA. Horizontal line designates shortest 20% of telomeres. Experiment performed by the group of Professor Shay.
3.3 teloC tncRNA inhibition results in replication stress-dependent cell death

3.3.1 ASO-mediated inhibition of teloC tncRNAs results in an S-phase accumulation and replication inhibition

A strong link between telomeric replication stress and ALT has recently emerged. To first query a possible connection between replication stress and tncRNA inhibition in ALT cells, I analysed the cell cycle of ASO-treated U2OS cells two days after transfection by staining them with propidium iodide (PI) for total DNA content, followed by flow cytometry analysis. While control and antiteloG ASO treatment have no effect on cell cycle relative to mock treatment, antiteloC ASOs induce an accumulation of cells in S-phase, reaching statistical significance when compared to each other treatment (p<0.05) (Figure 33). To further investigate this antiteloC ASO-induced S-phase accumulation, I briefly pulsed ASO-treated cells with BrdU prior to harvesting for flow cytometry analysis of BrdU incorporation as well as cell cycle distribution. BrdU is used as a marker for S-phase cells, as those that are actively replicating will incorporate BrdU. Interestingly, in antiteloC ASO-treated cells a population of cells displaying an S-phase amount of DNA, based on PI staining, but absent for BrdU incorporation appeared (Figure 34). This may suggest that teloC tncRNA inhibition leads to an event so catastrophic for the cell that global DNA replication is inhibited in response.

3.3.2 teloC tncRNA inhibition results in a replication-dependent apoptosis activation in ALT cells

To further characterize the relationship between teloC tncRNA inhibition and DNA replication in ALT cells, I sought to compare the impact of tncRNA inhibition on cell viability between cycling and arrested cells. The experimental scheme used is outlined in...
Figure 34. teloC tncRNA inhibition results in non-replicating S-phase cells
U2OS cells were treated with PS-LNA mixmer ASOs at 20nM, and two days later pulsed with BrdU, fixed, stained for PI and BrdU, and analyzed by flow cytometry. (a) fraction of total cells that display S-phase DNA content based on PI staining, but are negative for BrdU (b) example of PI (DNA content) and BrdU signal distribution of cells, red arrow points towards the population that is quantified in (a). n=2. Error bars represent s.e.m. *p<0.05

Figure 35a. I induced a G1-arrest in SAOS2 cells through 24 hours of serum starvation (Figure 35b, ~60% G1 arrest vs ~35% G1 cycling). After one day of serum starvation I transfected ASOs, and 24 hours later released cells into the cell cycle by providing them serum (cycling), or kept them arrested in serum-free media (arrested). Two days after release I monitored cell number relative to mock. While arrested cells suffered from ASO transfection per se, there seemed to be no specificity for ASO sequence, while the number of cycling cells was significantly affected only by antiteloC ASOs (Figure 35c).

Using the same experimental design outlined in Figure 35a, I monitored induction of apoptosis in real time following cell cycle release using the Incucyte system. I then mock transfected arrested SAOS2 cells, or transfected with control or antiteloC ASOs, and monitored the number of apoptotic cells every three hours after release. To measure relative apoptosis induction at each time point, I normalized the number of apoptotic cells of ASO-transfected cells on the number of apoptotic cells in mock treatment. In cycling cells the control ASO had no effect on apoptosis induction relative to mock treatment, while antiteloC ASOs induced a ~50% increase in apoptosis within 15 hours of treatment (Figure 36, left).
SAOS2 cells were arrested for one day by serum starvation, then transfected with PS-LNA mixmer ASOs at 20nM, one day later cells were either released into serum-containing media, or kept arrested in serum-free media, two days after release relative cell number was measured. (a) experimental scheme (b) cells were harvested after 24 hours of serum starvation for cell cycle analysis by flow cytometry. (c) cell viability was analysed in 6-well plates, see materials and methods. n=3. Error bars represent s.e.m. *p<0.05, **p<0.005 ***p<0.0005, ****p<0.00005.

SAOS2 cells were treated according to the experimental scheme outlined in figure 24a. Upon release into serum-containing media, the IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent was added, and acquisition in the Incucyte system began. Number of apoptotic cells relative to respective Cycling or arrested mock treatment for each time is shown.

Figure 35. Arrested SAOS2 ALT cells are not affected by tncRNA inhibition

Figure 36. Arrested SAOS2 ALT cells do not go into apoptosis following teloC tncRNA inhibition
3.3.3 Replication stress sensitizes ALT and non-ALT cells to teloC tncRNA inhibition

The causes and consequences of telomeric replication stress may be unique to the ALT pathway. Thus, I next sought to understand if tdilncRNA upregulation and necessity in ALT cells is linked specifically to the ALT pathway, or more generally to telomeric replication stress. As telomeres represent common fragile sites (CFS) (Sfeir et al. 2009), they are easily damaged when cells are exposed to chemicals that induce replication stress such as hydroxyurea (HU). To test this, I first induced replication stress in the ALT cell line U2OS and telomerase-positive cell line MG63 with HU and then measured tncRNA expression. U2OS (Figure 37a) and MG63 (Figure 37b) each displayed increased levels of both teloG and teloC tncRNAs following three days of exposure to HU at 0.25mM.

Possible upregulation of tncRNAs in MG63 in response to HU-induced replication stress suggests that tncRNAs may play a role at any telomere experiencing replication stress, not just ALT telomeres. It would follow, then, that non-ALT cells, such as MG63, may be sensitized to tncRNA inhibition under conditions of replication stress. I treated the ALT cell line U2OS, telomerase-positive cell line MG63, and telomerase-positive cell line RPE-hTERT with various concentrations of HU or aphidicolin (APH), which also induces replication stress, in combination with various concentrations of control, antiteloG, or antiteloC PS-LNA mixmer ASOs. In order to avoid complications of co-transfecting an ASO
together with a drug I treated cells with ‘naked’ ASOs (meaning without a transfection reagent) thus at higher concentrations than were used for transfections. I monitored relative cell number three days after treatment, and calculated the ‘Excess over Bliss’ of each ASO-drug combination to determine synergic, additive, or antagonistic effects on cell number (Price et al. 2003). Each cell line was treated with a range of concentrations, according to their relative resistance to antiteloC ASOs, because to calculate synergy between two treatments both must be used at concentrations that induce measurable effects regardless of specificity. The ‘Excess over Bliss’ analysis of a combination of drugs results in the percent of cells, with respect to control treatments, that is affected in excess over the expected response of the combined treatment. Briefly, this synergy calculation first involves calculating the predicted effect of the combination of two treatments on cells by looking at the effects of each treatment alone. This predicted effect is the outcome if treatments are additive: their effects are simply added together. Once the effect of each treatment alone is calculated, the predicted combined effect is compared to the observed combined effect, and the difference of the two can be calculated to determine synergism. If the observed effect of the combination is greater than the predicted effect, then the two treatments are synergistic (value>0); if the observed is equal to the predicted effect, the treatments are additive (value=0); if the observed is less than the predicted effect, the treatments are antagonistic (value<0).

HU sensitized both U2OS and MG63 to antiteloC ASOs with a peak in synergy at the highest antiteloC ASO concentration (10µM for U2OS, 50µM for MG63) and 0.25mM HU (Figure 38a,c peak of synergy right-most column, third concentration of HU from the top). Around this peak of synergy there were other similar, but slightly less synergic combinations of antiteloC ASO and HU at different concentrations, suggesting robust synergy. Meanwhile, most other apparently ‘synergic’ combinations of ASO and drug are likely due to noise. For example, U2OS treated with antiteloG ASOs at 1.11µM and 0.25mM HU (Figure 38a) seem to be synergic, but the fact that increasing antiteloG ASO concentrations results in a decreasing effect on cell viability at this particular concentration of HU suggests that there was some noise with those particular samples. Furthermore, if one of the two treatments alone does not reduce cell number, numbers yielded from synergy calculations will be based on noise, rather than true signal. Indeed, antiteloG ASOs have almost no effect on cell viability relative to mock in the absence of HU. As predicted effects of the combined treatments will be based on the observed individual effect, which, again, are small, and may be stochastic, the synergy calculations are not reliable.
Interestingly, APH did not induce sensitivity to ASOs in both U2OS (Figure 38c) and MG63 cells (Figure 38d), and RPE-hTERT was not sensitized by either HU or APH. The lack of induced sensitivity in RPE-hTERT is due to the lack of an effect of either ASO alone (Figure 38e), whereas the lack of induced sensitivity by APH in U2OS and MG63 is not quite as clear. Interestingly, at high concentrations of APH in MG63 cells there even seems to be an antagonistic effect with antiteloC treatments (Figure 38d). These results are likely explained by effects on the cell cycle of aphidicolin. I treated U2OS cells with vehicle, or two concentrations of HU (0.5mM and 1mM) and Aphidicolin (0.4µM and 1µM) then monitored cell cycle distribution 24, and 48 hours later by flow cytometry. While HU-treated cells displayed a more gradual, dose-dependent effect on cell cycle distribution (Figure 39a), at these ranges of APH cells were largely arrested, or almost entirely unaffected (Figure 39b). As I previously presented evidence for, ALT cells are not sensitive to ASOs when they are arrested. The outcome of this difference of effect on cell cycle is that HU seems to induce replication stress without completely arresting cells, thereby ‘allowing’ cycling cells to be targeted by antiteloC ASOs, while aphidicolin seems to either not induce replication stress at lower concentrations, or completely arrest cells at higher concentrations, thereby preventing ASOs from acting.

![Graphs showing cell cycle distribution](image)

**Figure 39. Cell cycle distribution following Aphidicolin and Hydroxyurea treatment**

U2OS cells were treated with vehicle, HU (a), or APH (b) at the indicated concentrations, and fixed 24 (left graphs) and 48 (right graphs) hours later for cell cycle analysis by flow cytometry.
**Figure 38. HU induces sensitivity to antitelοC ASOs in ALT U2OS cells and telomerase-positive MG63 cells**

Cells were treated with the PS-LNA ASOs control (ctrl), antitelοG (a-G), and antitelοC (a-C) or mock treated, in combination with HU (a, c, e) or APH (b, d, f) simultaneously at the indicated concentrations in duplicate, with duplicates spread between 2 plates. ASOs were administered without a transfection reagent. Cell number relative to untreated cells was monitored three days later (top). Synergy (bottom) values are color coded: higher synergy values correspond to a darker shade of red. (a,b) U2OS, (c,d), MG63, (e,f) RPEhTERT.
**Figure 38 (cont). HU induces sensitivity to antiteloc ASOs in ALT U2OS cells and telomerase-positive MG63 cells**

Cells were treated with the PS-LNA ASOs control (ctrl), antiteloG (a-G), and antiteloc (a-C) or mock treated, in combination with HU (a, c, e) or APH (b, d, f) simultaneously at the indicated concentrations in duplicate, with duplicates spread between 2 plates. ASOs were administered without a transfection reagent. Cell number relative to untreated cells was monitored three days later (top). Synergy (bottom) values are color coded: higher synergy values correspond to a darker shade of red. (a,b) U2OS, (c,d), MG63, (e,f) RPEhTERT.
Cells were treated with the PS-LNA ASOs control (ctrl), antiteloG (a-G), and antiteloC (a-C) or mock treated, in combination with HU (a, c, e) or APH (b, d, f) simultaneously at the indicated concentrations in duplicate, with duplicates spread between 2 plates. ASOs were administered without a transfection reagent. Cell number relative to untreated cells was monitored three days later (top). Synergy (bottom) values are color coded: higher synergy values correspond to a darker shade of red. (a,b) U2OS, (c,d) MG63, (e,f) RPEhTERT.
3.4 In vivo efficacy of antiteloC ASOs in a zebrafish xenograft model

3.4.1 Inhibition of teloC tncRNAs in vivo in a xenograft model in zebrafish causes ALT cell death

To investigate the possibility of in vivo efficacy of antiteloC ASOs in inducing cell death of ALT cells, we employed a zebrafish xenograft model in collaboration with Professor Marina Mione and her group at the University of Trento. ALT SAOS2 cells and ASOs were co-injected into the brain ventricles of Zebrafish larvae 2 days post fertilization. Larvae were harvested 1 day after treatment for analysis. Sections were stained with Hoechst to label nuclei, and nuclear morphology was examined to mark cells as apoptotic/necrotic or alive. Injection of antiteloC ASOs significantly increased the fraction of dead cells, with respect to control ASOs (Figure 40).

Figure 40. A SAOS2 xenograft model in zebrafish displays sensitivity to teloC tncRNA inhibition

SAOS2 cells and either control (LAC) or antiteloC PS-LNA mixmer ASOs were co-injected in the brain ventricles of zebrafish larvae two days post fertilization. One day later, larvae were harvested for cell death analysis. Dead cells were marked by specific Hoechst staining attributes (described in the materials and methods), and the proportion of dead cells out of total cells counted is reported. Error bars represent s.e.m. n=4. **p<0.005
3.5 Annex

3.5.1 Inhibition of dilncRNAs and DDRNAs with ASOs reduces repair efficiency of both HR and NHEJ at engineered genomic DSBs

We have previously established a role for DDRNAs and dilncRNAs in fuelling DDR focus formation following DSB induction (Francia et al. 2012, Michelini et al. 2017), but have not monitored consequential effects on repair by HR or NHEJ. To test these, I employed two cell lines containing inducible DNA double strand breaks, the repair of which results in fluorophore expression, thereby indicating the choice of repair pathway. We designed ASOs matching dilncRNAs and DDRNAs from around the breaks, and monitored repair choice following ASO treatment (ASOs listed in materials and methods, Table 2).

A simple scheme of how repair leads to fluorophore expression in these cell lines is depicted below (Figure 41a,b). In the first cell line, a modified U2OS called DR-GFP (Pierce et al. 1999), an I-SceI recognition site was introduced within a GFP gene, thereby introducing an early stop codon. Downstream of this GFP is a truncated GFP gene (not pictured) that can act as a donor for genetic material during repair by HR. Upon induction of I-SceI and DSB formation, if cells repair by HR using the downstream truncated GFP as the homologous sequence, a functional GFP will be formed and expressed. In the second cell line, a modified 293T called ‘Traffic Light Reporter’ (TLR) (Certo et al. 2011), an I-SceI recognition site was introduced within a GFP gene, thereby introducing an early stop codon. Downstream of this GFP cassette is an out of frame mCherry cassette. Upon cotransfection of a plasmid containing the I-SceI gene, and a plasmid harbouring donor material for repair of GFP by HR (plasmids not pictured), the cell must choose to repair by either HR, or NHEJ. These plasmids contain genes for fluorophore expression as well, in order to gate for those cells that received both. If the cell repairs by HR with the transfected template as a donor, a functional GFP gene will be formed and expressed, however if the cell repairs by mutagenic NHEJ, introducing a 2bp frameshift, the mCherry gene will be in frame and expressed. We designed ASOs against dilncRNAs from the DSBs as depicted in Figure 41c, and grouped ASOs against the sense strand on one side of the break with ASOs against the antisense strand on the other side of the break to form ‘Active ASO group 1’ and ‘Active ASO group 2’. ASOs were pooled in two separate groups to prevent them from binding to one another, while still targeting multiple dilncRNAs/DDRNAs. Three days after simultaneous induction of I-SceI and transfection with ASOs, cells were harvested for fluorophore expression analysis by flow cytometry. Relative to control ASO, both Active ASO groups 1 and 2 reduced repair efficiency by HR in both DR-GFP and TLR cells, as well as by NHEJ in TLR cells (Figure 41d).
Figure 41. ASO-mediated inhibition of repair by HR and NHEJ at engineered DSBs

(a) scheme of HR repair-induced fluorophore expression in DR-GFP cells (b) scheme of HR- and NHEJ repair-induced fluorophore expression in TLR cells (c) scheme of ASOs matching dilncRNAs and DDRNAs around the cut site (d) I-SceI expression was induced in DR-GFP cells with doxycycline, and in TLR cells by plasmid transfection, simultaneous to transfection with control (LAC sequence) or active PS-LNA mixmer ASO groups 1 or 2 (specific sequences in materials and methods Table 2). DR-GFP cells were transfected with 20nM ASOs, while TLR cells were transfected with 50nM ASOs. Three days later cells were fixed for fluorophore expression analysis by flow cytometry. TLR cells were gated for those expressing fluorophores from both plasmids. The fraction of total cells expressing GFP (DR-GFP and TLR cells) or mCherry (TLR cells only), normalized on Control ASO-treated cells is reported.
4 Discussion
4.1 Telomeric RNAs in ALT cells

4.1.1 ALT- and replication stress-induced telomeric transcription

We have previously shown that both teloG and teloC dilncRNAs and DDRNAs, collectively described here as tncRNAs, are upregulated upon telomere uncapping (Rossiello et al. 2017). In support of this notion, and in a context directly related to human physiology, we discovered a role for tncRNA induction in the progeric syndrome HGPS (Aguado et al. 2019). Specifically, we found that both expression of progerin in normal cells, and extended cell division of HGPS patient cells induces tncRNA transcription. Here we have shown that tncRNAs are also upregulated in ALT cells (Figure 9,10) and potentially also during replication stress (Figure 37). Given recent discoveries that ALT cells experience high levels of replication stress at their telomeres, and our observation here that tncRNA transcription may be triggered upon HU-induced DNA replication stress, endogenous replication stress is a prominent candidate for the source for tncRNA induction in ALT cells. These findings suggest that tncRNA induction is a general feature of telomere dysfunction.

Upregulation of tncRNAs in ALT cells is most apparent when comparing paired ALT and non-ALT cells (Figure 9), though it can also be appreciated when comparing non-paired ALT and non-ALT cells (Figure 10). This is likely explained by genetic differences between cells of unique origin resulting in differential telomere transcription regardless of TMM. The use of paired cell lines reduces this variability caused by distinct genetic makeups, allowing for TMMs to be more readily compared. The wide range of tncRNA induction magnitudes in ALT cell lines (Figures 9,10) is interesting, and could have multiple explanations, such as different levels of telomeric replication stress or different levels of baseline telomere transcription among cell lines. One possible cause for this could be differing telomere lengths. Longer telomeres seem to experience more replication stress than short telomeres (Rivera et al. 2017). Although the reason for this has not yet been elucidated, it may be due to the raw increase in telomeric repeats that increases the likelihood that DNA polymerases will encounter replication stress-causing structures. Shorter cell division times may also explain relative differences in telomeric replication stress, as cells with a short G1 phase, thus growing fast, experience more replication stress than slower growing cells with longer G1 phases (Ahuja et al. 2016). When quantifying tdilncRNA levels in unpaired ALT and non-ALT lines (Figure 10) ALT-specificity is less obvious, although still appreciable. Specifically, the ALT cell line G-292 displays very low levels of tdilncRNAs. Interestingly, G-292 cells grow slower than the other two ALT cell lines, SAOS2, and U2OS, to which it was compared (unpublished observations), thus their low levels of tncRNAs may be explained by slower divisions. Variable tncRNA induction between ALT lines, as well as in
responses to tncRNA inhibition, may also be explained by diversity in the ALT mechanism. Indeed, depletion of various proteins in different cell lines induces inconsistent changes in C-circle levels (Martinez et al. 2017), suggesting that ALT mechanisms differ between cell lines. Differences in telomeric compaction between ALT cell lines, possibly due to genetic or epigenetic factors, may also lead to differential replication stress: in the fission yeast *Schizosaccharomyces pombe* it was discovered that damage accumulates at heterochromatic regions, such as telomeres, during S-phase (Rozenzhak et al. 2010), and in human cells that telomeric compaction is seemingly increased at APBs (Osterwald et al. 2015), but decreased overall in ALT vs non-ALT cell lines (Episkopou et al. 2014). Compaction may also be affected by ALT telomere sequence heterogeneity (Lee et al. 2014), as it is linked to deficient TRF2 loading (Conomos et al. 2012), which, as described in the introduction, could lead to different levels of compaction. Other explanations for the wide range of tncRNA levels may be that longer telomeres allow for more transcription, or that different telomeric histone marks predominate in different cell lines, thereby affecting telomeric transcription. A comprehensive comparison between tncRNA levels and relative telomere length, TIFs, telomere compaction, and doubling time should be performed to identify factors in tncRNA expression level.

The G-rich telomeric RNA TERRA, transcribed from sub-telomeres, shares the same UUAGGG repeat sequence as teloG tdilncRNAs, however the 3′- end of TERRA contains the sequence of the subtelomere from which it was transcribed while teloG tdilncRNA does not, as they are transcribed from within the telomere. Thus, it could be that some published observations of TERRA that only consider its telomeric sequence are actually observations of teloG tdilncRNAs, and/or vice versa that some of our observations of teloG tdilncRNAs are actually of TERRA. However, short G-rich teloG RNAs (teloG DDRNAs), as well as both long and short C-rich telomeric RNAs (teloC dilncRNAs and DDRNAs, respectively), have not previously been described in mammals, thus as of now their induction here and in other systems (Rossiello et al. 2017, Aguado et al. 2019) is best explained by telomeric damage. Furthermore, teloG tdilncRNA expression was induced ~4-fold in progerin-expressing cells, while TERRA expression from specific subtelomeres was induced ~1.2- to ~1.6-fold (Aguado et al. 2019), suggesting that in these cells TERRA could only partially contribute to the observed increased teloG tdilncRNA levels.

To address difficulties in differentiating teloG tdilncRNA from TERRA transcripts, taking inspiration from the ‘splinted ligation’ technique to quantify low abundant miRNAs
guaranteeing that the RNA amplified will also amplify TERRA, the adapter sequence is used as one of the two primers for 3′ RT.

Figure 42. Splinted-ligation RT-qPCR for specific quantification of teloG tdilncRNAs

Splinted ligation: Cellular RNA, containing both TERRA species with 3′ subtelomeric sequences (black) and 3′ UUAGGG repeats (blue), and teloG tdilncRNA species of full UUAGGGn telomeric repeats (blue), is hybridized with a Bridge oligonucleotide whose 3′ end is complementary to UUAGGGn telomeric repeats (blue), and whose 3′ end is complementary to a unique sequence (orange), and a Unique sequence oligonucleotide that is complementary to the 3′ end of the bridge (orange). cDNA library preparation: reverse transcription with teloC primers, containing CCCTAA repeats, to create cDNA libraries of both 3′-modified teloG tdilncRNAs and TERRAs. Selective amplification of teloG tdilncRNAs: unique primers will amplify 3′-modified RNAs only, while teloC primers will amplify all telomeric species; only 3′-modified RNAs will be exponentially amplified.

(Maroney et al. 2008), a possible approach is described above (Figure 42). Briefly, cellular RNA is incubated with a “bridge” oligo, the splint, whose 3′-end is complementary to the 3′ end of teloG tdilncRNAs and whose 3′-end is of a unique sequence, along with another “unique” oligonucleotide whose sequence is complementary to the unique sequence of the 3′ end of the bridge. The bridge oligo will bind the adapter oligo on its 3′-end, and on its 3′-end to the 3′-end of teloG tdilncRNAs, then ligase covalently joins the 3′-end of the unique to the 3′-end of teloG tdilncRNAs. Following strand-specific RT of all G-rich RNAs, which will also amplify TERRA, the adapter sequence is used as one of the two primers for qPCR, guaranteeing that the RNA amplified is 3′ modified, and thus contained pure 3′-UUAGGGn.
repeats prior to splinted ligation, thus excluding TERRA. Importantly, the success of this approach relies on TERRA not being nucleolytically processed. Although it has not yet been reported, removal of the subtelomeric 3’-end of TERRA would make it indistinguishable from teloG tdilncRNAs in this assay, complicating their differentiation. Only experimental tests will demonstrate the use of this approach in distinguishing teloG tdilncRNA levels from TERRA levels.

4.1.2 ASOs as a tool to study the role of telomeric RNAs in ALT cell viability

In order to investigate the role of tncRNAs in ALT I have used ASOs. In our first study on tncRNAs we were able to use other methods such as depletion of RNA pol II and tncRNA processing factors such as DICER and DROSHA, or RNaseA treatment of fixed cells, in order to interrogate a role of tncRNAs in signalling for telomeric DDRs (Rossiello et al. 2017). As depletion of these factors is toxic for cells when maintained for days (unpublished observations), here we used ASOs to target tncRNAs. Others have also used ASOs to interrogate telomeric RNA function, specifically targeting TERRA (Deng et al. 2009, Chu et al. 2017, Avogaro et al. 2018), though to our knowledge none have investigated or reported effects on viability, nor targeted C-rich telomeric RNAs.

Interestingly, while almost all antiteloC ASOs tested reduced ALT cell number, only two of the six 8nt PS-LNA Mixmer Short antiteloC ASOs reduced ALT cell number (Figure 16). As the explanation given previously, the biggest contributor to this differential effect between short and long ASOs is likely size: at similar concentrations the longer 21nt ASO ‘covers’ more of the target RNA than the shorter 8nt ASO. However, this does not

<table>
<thead>
<tr>
<th>Short antiteloC-#</th>
<th>Sequence</th>
<th>Reduced U2OS cell number?</th>
<th>G-content</th>
<th>Possibility of self annealing</th>
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<td>-</td>
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Table 4. Analysis of sequence, effect, G-content, and self-annealing possibilities of PS-LNA mixmer Short antiteloC ASOs

ASO name, sequence, effect on U2OS cell number, G-content, and possible self-annealing alignments. Mismatches in self-annealing pairs are highlighted in red.
adequately explain differences among 8nt ASOs. An analysis of these short ASO sequences is listed in Table 4. The Short antiteloC-4 ASO, which appreciably reduced U2OS cell number at both 20nM (Figures 16, 17) and to a greater extent 60nM (Figure 18), contains the most guanosines, which makes for a higher bond strength than thymidine or adenosine. The Short antiteloC-5 ASO contains the same number of guanosines, and indeed it was the only other ASO to reduce U2OS cell number following three transfections over 10 days, although did not reach statistical significance (Figure 16). A study of DNA oligonucleotide pair stability revealed the following, decreasing order of stability, highest listed first: GC>CG>TA>AT>GT>AG>GG>TG>TT=GA>TC>AC>CT>AA>CA>CC (Pan et al. 2006). Short antiteloC-1,2,3, and -6 may dimerize based on stable Watson-Crick base pairing at the end of the oligo, and at least once internally, combined with stable mismatches of T-G and G-G, while Short AntiteloC-4, and 5 are incapable for forming a Watson-Crick base pair at their ends. Thus, a higher ASO concentration of these short ASOs may be required in order to overcome self-annealing and weak binding strength, and thereby reduce ALT cell number. Further experimental exploration of these differences is required to confirm these hypotheses.

It would be informative to examine tncRNA levels following ASO treatment. However, as mixmer ASOs inhibit target RNAs through steric hindrance, the level of ASO targets is not expected to change, and as such has not been investigated. Gapmer ASOs, on the other hand, function by degrading target RNAs, and as such investigating their effect on ALT features as well as tncRNA levels would be revealing. There may be caveats to such an approach, however, such as the possibility that ASOs can interfere with either reverse transcription or qPCR by binding to target sequences and blocking polymerases, complicating potential results.

The most likely target of antiteloC ASOs in inducing apoptosis in ALT cells is teloC tncRNA, because: cells that are specifically sensitive to antiteloC ASOs, possibly following HU treatment (Figure 38) or due to engagement with the ALT pathway (Figures 12-21), express elevated levels of tncRNAs (Figures 37, 9, 10 respectively); in other settings, roles of tncRNAs have been probed by both ASOs and independent methods, yielding similar or consistent results (Deng et al. 2009, Chu et al. 2017, Rossiello et al. 2017, Avogaro et al. 2018, Aguado et al. 2019); and ASOs display a high affinity for RNA, which they are designed to target, and have been successfully used to target pathological RNAs in physiological settings with few side effects (McClorey et al. 2015, Scoles et al. 2019). However, below I explore possible off-target effects. First, as described in section 3.1.8, antiteloC ASOs have the potential to form G4s, and G4-forming oligonucleotides display anti-cancer properties, which, importantly, were not specific for ALT cells, on the contrary
they may actually be specific for telomerase-positive cells due to inhibition of telomerase expression (Chhabra et al. 2018). While PS-LNA mixmer antiteloC ASOs do form G-quadruplexes, PS-LNA mixmer short antiteloC-4 and PS-2’-O-Me mixmer antiteloC ASOs do not (Figures 25, 26). Cell number reduction following antiteloC ASO treatment is therefore unlikely to be due to G-quadruplexes as PS-2’-O-Me antiteloC ASOs (does not form G-quadruplexes) reduce cell number of U2OS cells to an even greater degree than PS-LNA mixmer antiteloC ASOs (does form G-quadruplexes) (Figure 21), and when 8nt PS-LNA mixmer short antiteloC-4 ASOs (does not form G-quadruplexes) are transfected at 3-fold higher concentrations, thus with comparable RNA coverage (as explained in section 3.1.2) with respect to 21nt PS-LNA mixmer antiteloC ASOs (does form G-quadruplexes), they reduce cell number to similar extents (Figures 18, 19).

It was also discovered by Professor Barbara Gilchrest and her group that ‘t-oligos,’ DNA oligonucleotides with almost complete homology to the G-rich strand of telomeres, induce cell death by triggering a DDR, which the authors claim is due to the oligo’s homology to telomeres. T-oligo-induced cell death was not ALT-specific, as many telomerase-positive lines responded (Sarkar et al. 2011). Nonetheless, as ASOs used here to target teloC tncRNAs are indeed perfectly homologous to the telomere, we considered the possibility that they act as the t-oligos described above. However, in elucidating this mechanism, they found that nuclease-resistant oligonucleotides do not evoke the same response, specifically using phosphorothioate modifications (Eller et al. 2006). As ASOs that we used are designed to be resistant to degradation, specifically with the phosphorothioate (PS) modification, and t-oligos were found to act on non-ALT cells, it is unlikely that antiteloC ASOs are acting as t-oligos.

Finally, as ASOs are already widely used in clinics to target pathological RNAs (McClorey et al. 2015), it is unlikely that they cause side effects by targeting DNA. Nonetheless, to investigate this possibility, as well as to explore alternatives to ASOs, we are currently investigating other technologies such as siRNA. Although siRNA is classically considered to function only in the cytoplasm it has been demonstrated that siRNA machinery is present and active in the nucleus (Gagnon et al. 2014), providing for the possibility of targeting telomeric RNAs with siRNAs. As with ASOs, there are certain complications that we foresee accompanying this approach. siRNAs are typically purchased as double-stranded RNAs, and once delivered to cells the antisense strand is loaded into the RNA-induced silencing complex (RISC) for sequence-specific RNA degradation. The main factor guiding strand-specificity of siRNA loading into RISC is the thermodynamic stability of the 3’-end (Schwarz et al. 2003, Noland et al. 2011). Despite this, siRNAs designed with one strand containing a less thermodynamic 3’-end, therefore promoting it’s RISC loading, are capable
of degrading both sense and antisense targets if they are present (Wei et al. 2009). This presents the problem that any double-stranded siRNA against telomeric sequences may have the capability of targeting both teloG and teloC tncRNAs, regardless of which the intended target was. Interestingly, inhibiting TERRA by siRNA has already been shown to reduce U2OS cell number (Deng et al. 2009). Considering only TERRA as the siRNA target, this contrasts with our results that antiteloG ASOs have no effect in U2OS cells (Figures 12-21). Although Deng et al. did show that TERRA was degraded, it could be that the effect on viability of U2OS cells was actually an effect of teloC tncRNA degradation which was not studied, or reported. In support of this notion, antiteloG gapmers, which normally function by degrading target RNAs, had no effect on U2OS cells at any concentration tested (Figure 21). However, it has recently been shown that ASO gapmers harbouring the chemical modification 2′-deoxy-2′-fluoroarabinonucleotide (FANA) inhibit target RNA function both through steric hindrance, and by inducing degradation by RNaseH (Takahashi et al. 2019), providing for the possibility that telomeric gapmer ASOs may also function through steric hindrance rather than targeted degradation. Given these observations, in the context of targeting telomeric RNA to reduce U2OS cell number, it would seem that either: siRNAs against TERRA are indeed targeting only TERRA, and that steric hindrance of telomeric RNAs is functionally distinct from degradation, with gapmers not inducing TERRA degradation; or more likely that siRNAs against TERRA are targeting both strands of telomeric RNA, and effects on viability seen by Deng et al. are due to teloC tncRNA degradation.

We are also investigating CRIPSR-Cas technologies as an alternative method of tncRNA inhibition. The first observation of RNA-targeted CRISPR-Cas combinations was the type VI RNA-guided ribonuclease C2c2 protein from the bacterium Leptotrichia shahii (Abudayyeh et al. 2016), now termed Cas13. Further characterization of Cas13d revealed ~90% efficiency and high specificity in mRNA knockdown in the absence of DNA cleavage (Konermann et al. 2018), making it an appealing method to inhibit tncRNAs in a sequence-specific manner without the possibility of targeting DNA.

4.1.3 Sensitivity of ALT cells to antiteloC ASOs, but not antiteloG ASOs

ALT cells consistently displayed sensitivity to antiteloC ASOs, but not to antiteloG or unrelated ASOs, even when distinct chemistries over a range of concentrations were employed (Figure 21). This is curious, as both teloC and teloG tncRNAs are upregulated both in ALT (Figures 9, 10) and putatively refollowing replication stress induction (Figure 37), and both strands of tncRNA play relatively equal roles at uncapped and dysfunctional telomeres (Rossiello et al. 2017, Aguado et al. 2019). Additionally, DSBs at non-telomeric
loci depend on both sense and antisense strands of DDRNAs and dilincRNAs to fuel the local DDR, as well as repair (Michelini et al. 2017) (Figure 41). Our first hypothesis to explain this was that targeting teloG tncRNAs with ASOs may be non-toxic if TERRA “absorbs” the majority of antiteloG ASOs making them unavailable to target teloG tncRNAs. We sought to test this hypothesis by treating with ASOs a cell line displaying strongly reduced TERRA expression, which we thought may reveal sensitivity to antiteloG ASOs. Although we did find a seemingly suitable cell line, expressing ~80% less TERRA than the control cell line (Figure 22a), it was not sensitive to antiteloG ASOs (Figure 22b). Although it is possible that in this cell line TERRA levels were still sufficient to absorb antiteloG ASOs, this result seems to point towards a unique role for teloC tncRNAs in ALT cells and during replication stress. It may be that damage caused by replication stress results in structures different from those formed at DSBs, or at uncapped and dysfunctional telomeres, thereby more heavily depending on the teloC strand of dilncRNAs/DDRNAs. These possibilities are explored in the next section.

4.1.4 Possible roles of damage-induced telomeric RNAs in ALT cells and during replication stress

As the role of dilncRNAs/DDRNAs at DSBs, and tncRNAs at uncapped and dysfunctional telomeres, the role of tncRNAs at ALT telomeres and during replication stress is most likely to propagate the DDR signal by recruiting specific proteins. Indeed, inhibition of dilncRNAs and DDRNAs from an engineered DSB reduced repair efficiency by both HR and NHEJ (Figure 41), suggesting insufficient recruitment of repair factors. Inhibition of a proper telomeric replication stress response as a consequence of antiteloC ASO treatment is supported by the observations that antiteloC ASO-treated ALT cells accumulate in S-phase (Figure 33), display reduced BrdU-incorporation (Figure 34), and do not display growth defects nor activate apoptosis when arrested (Figures 35,36). The inhibition of teloC tncRNA results in cell death of ALT cells specifically (Figures 12-19), and HU-treated cells regardless of TMM (Figure 38), suggesting that they may be essential during telomeric replication stress responses. One would assume that apoptosis induced by inhibition of telomeric replication stress signalling or repair would result in a noticeable telomeric phenotype, such as sudden loss or induction of fragility. On the contrary, inhibition of tncRNAs does not seem to result in major changes to telomere fragility, fusion, or loss (Figure 30a,b,c), nor to T-SCE (Figure 28) suggesting that either cells induce apoptosis and die prior to mitosis, thereby removing affected cells from the analysed population, or that only few affected telomeres per cell are sufficient to induce apoptosis. ALT-specificity in cell death following teloC tncRNA inhibition is likely explained by increased loads of
replication stress at ALT telomeres, evidenced by the focal accumulation of DDR factors at ALT telomeres in APBs (Wu et al. 2003, Draskovic et al. 2009, Osterwald et al. 2015). What the specific replication stress structures are that induce tncRNA expression and which proteins are recruited in a tncRNA-dependent manner are as of yet unknown, but are of paramount interest. Below I explore several possibilities. Some experiments that were analysed here and were considered to develop a working model must be repeated in order to be statistically sound, however models and conclusions displayed here were generated with the data presented.

Telomeric replication stress events can result in fork reversal, the process in which nascent DNA dissociates from its template, anneals together and protrudes, thereby forming a structure resembling a holiday junction (Sogo et al. 2002), and in fork collapse, resulting in a DSB (Saintigny et al. 2001). At the telomere, where replication generally proceeds unidirectionally (Miller et al. 2006, Drosopoulos et al. 2012), fork reversal would result in a protrusion resembling a single-sided telomeric DSB with a G-rich 3′-end. Thus, it may be that MRN recognizes the tip of this DNA, as we have shown previously (Michelini et al. 2017), and recruits RNA Pol II, thereby inducing teloC tncRNAs, which may go on to promote repair of the reversed fork (Figure 43, left). Although not quantified in the publication, reversed fork protrusions seem to be less than 2kb (Sogo et al. 2002), thus transcription of teloG tncRNAs in the opposite direction may be inhibited by proximity of the holiday junction, reducing RNA pol II loading. Alternatively, as teloC tdilncRNAs will be transcribed from a free DNA end in this model, typically more abundant than dilncRNAs transcribed towards free DNA ends (Michelini et al. 2017), they may more stably form RNA-DNA hybrids upon telomeric replication stress. ALT cells carefully regulate RNA-DNA hybrids at their telomeres (Arora et al. 2014) and we have shown that RNA-hybrids induced at DSBs promote repair by HR by recruiting HR proteins. Specifically, we demonstrated that BRCA1 can directly recognize DNA:RNA hybrid, and I demonstrated that ASO-mediated inhibition of these hybrids reduced DNA repair by HR in an established cell-based assay (D'Alessandro et al. 2018) (Figure 41). By interfering with teloC tncRNA-DNA hybrid formation at ALT telomeres, DNA repair may be impaired, leading to apoptosis. Another possible scenario that may explain a specific role for teloC tncRNA species at ALT telomeres lies in a model recently proposed to explain how replication stress is resolved at ALT telomeres (Zhang et al. 2019). Zhang et al. suggest that at ALT telomeres reversed forks are processed by SLX4 into one-ended DSBs, with a G-rich 3′-end (Figure 43, right). As above, MRN may recognize this one-ended DSB, recruiting RNA Pol II, and
Figure 43. Model: teloC tncRNAs protect stalled replication forks

A replication fork entering the telomere may stall, resulting in fork reversal. Reversed forks can be recognized by MRN, or resolved by SLX4 into two one-ended breaks. MRN can recruit RNA Pol II to the reversed fork or one-ended breaks, provoking teloC tncRNA transcription (teloG tncRNA transcription not pictured here), which then promotes recruitment of fork rescue proteins, and thus restart, allowing for cell survival. However, in the presence of antiteloC ASOs teloC tncRNA function is inhibited, and fork rescue protein recruitment may be deficient, resulting in fork collapse, C-circle generation, and cell death.
thereby inducing transcription from the DNA end resulting in teloC tncRNAs, while teloG tncRNAs may be transcribed less due to protein blocks on the DNA left over from the holiday junction or replication, or due to the stronger induction of dilncRNA from DNA ends that we have previously observed (Michelini et al. 2017). Zhang et al. propose that RAD51 mediates strand exchange following SLX4-mediated DSB formation at reversed replication forks in ALT telomeres, thereby promoting replication restart, and indeed it has been shown that RAD51 mediates fork restart following reversal (Petermann et al. 2010). We have seen that dilncRNAs formed at genomic DSBs promote the recruitment of HR factors such as RAD51 (D'Alessandro et al. 2018), providing for the possibility that teloC tncRNAs act at stalled replication forks to recruit RAD51, and/or other factors, to promote replication restart. In antiteloC ASO treated ALT cells, then, teloC tncRNAs would be unable to recruit RAD51, or other repair factors, resulting in a toxic holiday junction intermediate and eventual cell death. In their model, Zhang et al. suggest that fork collapse promotes C-circle production, which we have shown may also be a consequence of antiteloC ASO-mediated inhibition of teloC tncRNAs (Figure 29), supporting the possibility that teloC tncRNA inhibition blocks processing of reversed forks, leading to fork collapse and C-circle production.

In line with strand-specific roles for tncRNAs, preferential elongation of lagging strands at ALT telomeres has been proposed (Min et al. 2017). Their explanation for this unilateral strand elongation relies on HR-factor recognition of exposed ssDNA on the 3′ end of the lagging strand, which is generated due to the incomplete fill-in of the lagging strand following telomere replication (Figure 44, left). An alternative explanation may be that upon replication fork collapse and one-ended DSB generation the leading strand is almost fully double-stranded and thus does not trigger a DDR, while the lagging strand will be partially single-stranded, thereby triggering a DDR, or that the two ends both trigger a DDR, but are differently processed due to the presence of ssDNA on the lagging strand (Figure 44, right). For both of these possibilities, MRN may recognize the dsDNA-ssDNA junction, which has been previously shown (Duursma et al. 2014), and thereby facilitate recruitment of RNA Pol II (Figure 44). As described above, transcription from the end of this template would result in teloC tncRNAs, which then may promote recruitment of repair factors to the lagging strand, thereby promoting strand invasion of other telomeres or C-circles, and thus ultimately leading to elongation.

We have also shown that teloC tncRNA decreases APBs (Figure 27) and may increase T-SCE events (Figure 28). The opposing roles of BLM/the BTR complex and SLX4/SLEX/ERCC4 at ALT telomeres (Sobinoff et al. 2017, Panier et al. 2019) is somewhat
similar in that BLM promotes APBs and reduces T-SCE, while SLX4 reduces APBs and promotes T-SCE. Inhibition of teloC tncRNAs seems to shift the equilibrium away from

Figure 44. Model: teloC tncRNAs promote preferential elongation of lagging strands

The ssDNA patch on the lagging telomere strand left over at the end of replication, or as a product of fork collapse can stimulate MRN localization, thus recruiting RNA Pol II. Transcription away from the telomere end results in teloC tncRNA expression, which in turn may facilitate strand invasion by recruiting HR factors, thereby allowing for elongation of the lagging strand, and cell survival. In the presence of antiteloC ASOs, however, teloC tncRNA function is inhibited and thus does not recruit HR factors, preventing elongation of the lagging strand and cell survival.
dissolving (BLM/BTR) to resolving (SLX4/SLX1/ERCC4) of intermediates, suggesting that teloC tncRNAs may recruit BLM, the BTR complex, or some other important factor such as POLD3.

Importantly, these models suggest that teloC tncRNAs are highly induced at ALT telomeres and telomeric replication stress sites while teloG tncRNAs are also induced, but possible to a lesser extent. In this case, ‘teloG tdilncRNA upregulation’ observed in ALT and during replication stress may partially be due to TERRA upregulation, while teloG tDDRNA induction might come from TERRA/teloG tdilncRNA and teloC tdilncRNA pairing and processing. Characterization of teloG tncRNAs 3’-end to distinguish them from TERRA, and quantification of TERRA by subtelomere-specific RT-qPCR will allow us to investigate these possibilities. Or, if teloG and teloC tdilncRNAs are equally induced at ALT telomeres, strand specificity for tncRNA roles may also be explained by teloC tdilncRNAs more stably forming RNA-DNA hybrids upon replication stress than teloG tdilncRNAs and thereby recruiting HR factors and promoting cell viability, as described above.

An alternative explanation for strand-specificity of tncRNA relevance in ALT and at telomeres experiencing replication stress comes from our recent discovery that dilncRNAs also fuel DDR focus formation by driving phase separation at the DSB (Pessina et al. 2019). Briefly, phase separation entails the creation of a cellular compartment with distinct physical properties that set it apart from the rest of the nucleoplasm or cytoplasm. Phase separation was first described in a study of P granules (Brangwynne et al. 2009), and then again later as a general rule to explain how nuclear bodies recruit and retain their components, specifically showing that PML nuclear bodies display phase separation properties (Banani et al. 2016). Interestingly, a role for phase-separation at ALT telomeres was also suggested (Min et al. 2019), in which the induction of phase-separation events at ALT telomeres mimicked APB formation, and induced ALT phenotypes. There is also unpublished evidence, available on BioRxiv, that endogenous APBs display liquid behaviour as well (Zhang et al. 2019). Given this liquidity of APBs, and the fact that they frequently contain DDR proteins (Tarsounas et al. 2004, Jiang et al. 2005, Zeng et al. 2009), it could be that as dilncRNAs fuel DDR focus formation through phase separation, teloC tdilncRNAs also fuel APB focus formation through phase separation. If this is the case, then teloG and teloC tdilncRNAs may be equally induced upon damage, but since teloG species form G-quadruplexes they would be less likely to contribute to phase separation than the unstructured teloC tncRNA, and the targeting of teloC tncRNAs with rigid ASOs would prevent them from creating a phase separated environment, resulting in decreased APBs (Figure 27).
A much more simplistic possibility to explain strand specificity in tncRNA roles at ALT and replication-stressed telomeres is that teloC tncRNAs may recruit some specific replication stress-responding proteins that do not bind to teloG tncRNAs. To test this hypothesis, it may be possible to characterize the proteome of teloG and teloC tncRNAs in ALT cells, and cells experiencing replication stress.

All of these possible mechanisms by which ASO-mediated inhibition of teloC tncRNAs induces cell death specifically in ALT cells and those challenged with replication stress need to be further investigated prior to drawing solid conclusions. Testing for tncRNA-dependent telomere localization of various DNA replication stress-, DDR-, and ALT-related proteins are of particular interest, but has not yet been performed due to time constraints. In order to further investigate the link between tncRNAs and ALT, we also wish to monitor the effects of tncRNA inhibition on recently discovered ALT features, such as BITS, telomeric MiDAS, and G2 telomeric DNA synthesis.

An alternative approach to study the role of tncRNAs in ALT cells is to expand synergy tests to other compounds with well-defined effects on telomeres, and measure tncRNA induction following treatment. For example, PARP inhibition was shown to induce cell death of cells experiencing telomeric crisis (Ngo et al. 2018), suggesting a critical role for PARP at critically short telomeres, which may appear as damaged to the cell, thus its combination with tncRNA inhibition in ALT cells may shed light on the latter’s function. Finally, further characterization of teloG tncRNAs to distinguish them from TERRA will allow us to better understand why antiteloG ASOs have no effect on ALT cells and cells experiencing telomeric replication stress.

4.2 Common fragile sites and non-coding RNAs

The relationship between transcription and replication stress is most commonly studied as the former causing the latter, rather than vice versa, but here I present evidence that replication stress may induce tncRNAs (Figure 37). The importance of the induction of this tncRNA, specifically teloC tncRNA, upon replication stress is outlined by their role in maintaining cell viability. This opens up the possibility that dilncRNAs and DDRNAs are induced at all sites of replication stress and function to support resolution of the event. In support of this it was recently discovered that the endoribonucleases DICER and DROSHA, involved in miRNA and DDRNA biogenesis, maintain genomic integrity following replication stress (Fragkos et al. 2019). Specifically, it was found that under conditions of replication stress and in the absence of DICER, CFS breakage, an S-phase arrest, and an S-phase checkpoint were induced, and 53BP1 foci accumulated most likely due to build-up of
unrepaired damage, and DROSHA depletion similarly increased CFS breakage and S-phase checkpoint activation. However, no mechanism for the roles of DICER and DROSHA in the replication stress response was suggested. Given the data shown here, I propose that sites of replication stress may be transcribed to produce dilncRNAs, similar to DSBs, which are then processed by DICER and DROSHA into DDRNAs, that in turn fuel the DDR associated with the replication stress and promote a timely repair. Monitoring dilncRNA and DDRNA levels from CFSs would be of interest not only to further characterize the replication stress response, but also to learn more about CFS-associated cancers. In particular, the best characterized CFSs are FRA3B and FRA16D, which are found in the tumor-suppressor genes FHIT and WWOX, respectively (Ohta et al. 1996, Bednarek et al. 2000). These regions are characterized by several break points that give rise to specific rearrangements frequently observed in cancer, or following replication stress (Durkin et al. 2008). Sequencing and quantification of replication stress-induced RNAs transcribed in these regions or at other fragile sites, particularly at the break points which are presumably the regions broken during replication stress and tumor progression, may reveal novel targets for cancer treatment.

4.3 Telomeric RNAs as a novel target for tumor therapies

ALT cancers typically have poor outcomes (Costa et al. 2006, Matsuo et al. 2009, Matsuo et al. 2010, Venturini et al. 2010, Pezzolo et al. 2015), and no targeted treatment is available yet. However, great strides have been made in our understanding of ALT in recent years, and putative targets have been identified such as PCAF (Bakhos-Douaihy et al. 2019), TSPYL5 (Episkopou et al. 2019), and FANCM (Silva et al. 2019). Here, we show that ALT cells express elevated levels of tncRNAs, and that ASO-mediated inhibition of teloC species results in ALT-specific cell death. Interestingly, HU synergizes with antiteloC ASOs in ALT cell lines, and sensitizes non-ALT cell lines to antiteloC ASOs (Figure 38), suggesting that tncRNA inhibition could possibly be combined with currently in-use cancer treatments that function through exacerbating or targeting cancer-associated replication stress in both ALT and non-ALT tumors.

The use of ASOs in clinics to target ncRNAs associated with cancer (reviewed in (Matsui et al. 2017)) suggests low toxicity and high efficacy, making it easier to move ASO-based treatments from bench to bedside. The only other physiological context in which we have observed upregulation of tncRNAs to date is progeria (Aguado et al. 2019), and in this case their targeting also proved beneficial. Thus, it seems likely that targeting tncRNAs is not associated with toxicity. With this characterization of the role of tncRNAs at ALT
telomeres we would like to suggest the addition of ASO-mediated inhibition of teloC tncRNAs to the small, yet growing list of potential ALT-targeting strategies for cancer therapy.
References


Appendix

**Figure 45. ASO running properties on denaturing and native SDS-PAGE (UNCROPPED)**

The indicated ASOs and DNA markers were run on a 20% SDS-PAGE at 5µM in 50 mM Tris-HCl, pH 7.4, 100 mM KCl under (a) denaturing 7M urea or (b) native conditions. (c) list of oligonucleotides tested, of which lanes 2, 7, 8, 10, and 11 were cropped and displayed in Figure 25.